EFFECTS OF ANDROGENS ON IMMUNITY TO SELF AND FOREIGN

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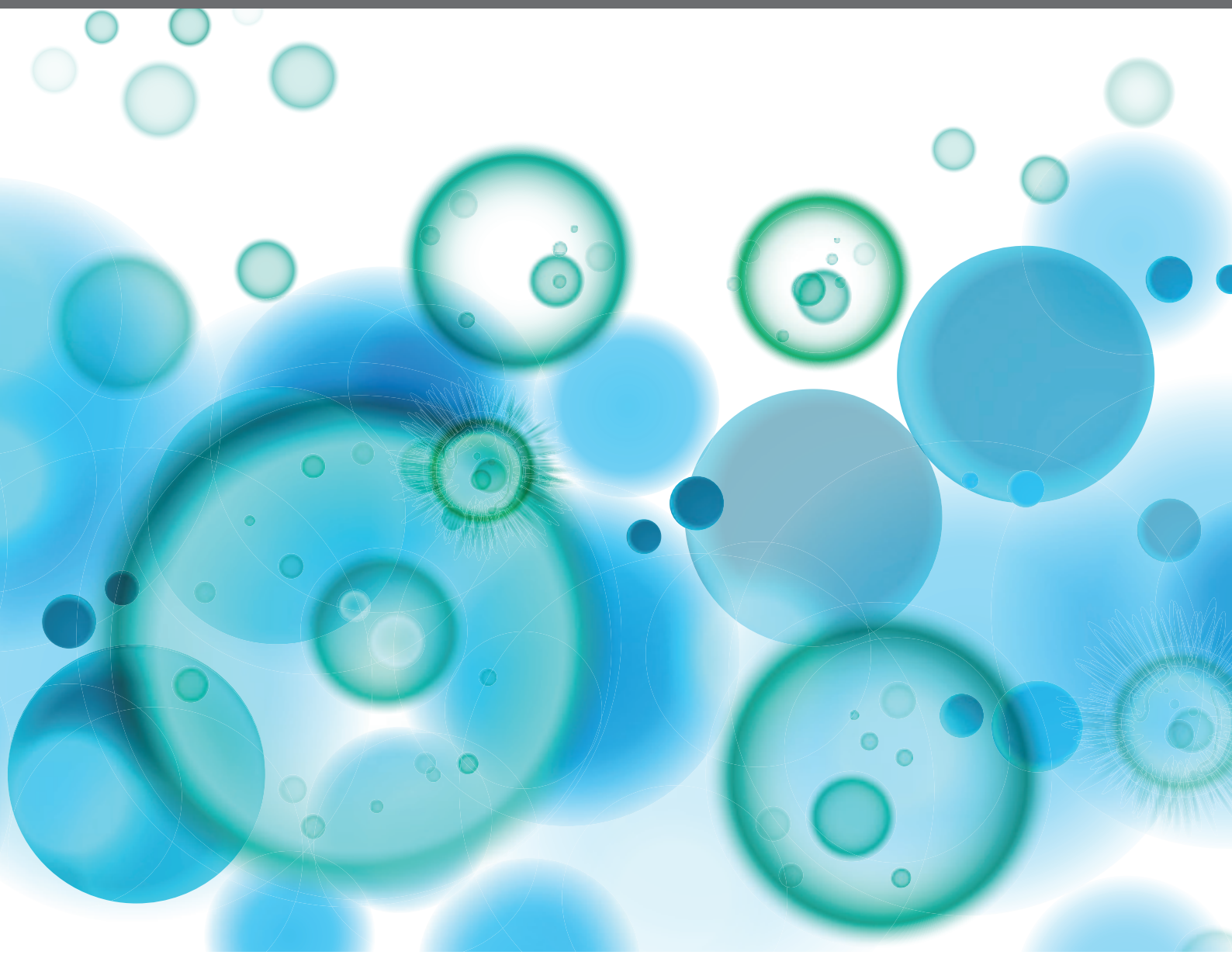

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EFFECTS OF ANDROGENS ON IMMUNITY TO SELF AND FOREIGN

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Table of Contents

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EDITORIAL
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Editorial: Effects of Androgens on Immunity to Self and Foreign

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Keywords: androgen, sex differences, immune cells, cancer, infection, autoimmunity

Editorial on the Research Topic

Effects of Androgens on Immunity to Self and Foreign

It is well established that male and female sex hormones regulate our immune system, leading to sex-specific differences in the outcome and manifestation of many diseases. As such, females often show superior protection against infections and some tumors and elaborate a better vaccination response due to immune-modulating effects of estrogens and progesterone, yet this enhanced immunity is coupled with an increased incidence of autoimmune diseases [reviewed in (1, 2)]. While anecdotally known since ancient times, only over the past couple of decades has the immunosuppressive effect of male sex hormones [testosterone, dihydrotestosterone (DHT)] been recognized. Testosterone and its most active metabolite DHT bind cytosolic or membrane-bound androgen receptors (ARs) to either directly or indirectly modulate gene transcription. ARs are expressed by many cells in the body including the majority of developing and some mature immune cells. Investigations of the role of testosterone in infectious diseases, autoimmunity and cancer have revealed direct effects of testosterone on immune cell development and function, with a general outcome of immunosuppression.

Although it remains questionable if sex hormones can be effectively used as therapeutic agents to treat diseases due to the many potential side-effects of such treatment, an understanding of testosterone-regulated cellular and molecular pathways may facilitate the development of innovative intervention strategies involving non-steroidal selective AR modulators that mediate tissue-specific activation of ARs (3), or the identification of cell-specific downstream signaling components that could be therapeutically targeted by gene manipulation strategies or small molecule inhibitors. In this article collection, authors from all over the world present new data and discuss the cellular and molecular impact of androgens in a variety of diseases including infections, cancer and autoimmunity.

In recognition of the potential of AR activity to modulate disease incidence and prevalence, the scientific community is actively investigating mechanisms by which testosterone regulates immune cells. For example, further investigations into the testosterone-driven positive regulation of Foxp3 and regulatory T cells, or into the skewing of CD4+ and CD8+ responses, may lead to the identification of specific drugs that can drive *de novo* development of T cell subsets for treatment of autoinflammatory diseases. The effect of androgens on T cells is reviewed in the setting of autoimmune liver diseases by Henze et al. Similarly, it has been shown that androgens regulate both myeloid cell differentiation and function. In this collection, a role for androgens in myeloid cell

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development and differentiation is shown by Consiglio and Golnick and Hreha et al., and reviewed in the setting of lupus by Jones and Jorgensen, while functionally, the production of testosterone-regulated lipid mediators by myeloid cells is discussed in the context of inflammation resolution by Pace and Wertz. We are hopeful that these and similar observations will drive *de novo* discovery of reagents capable of controlling testosterone-driven T cell and myeloid cell differentiation and trafficking patterns in infectious diseases, cancer and autoimmunity.

In addition to direct effect on immune cells, a role for androgens in driving tissue-specific differences in inflammation susceptibility are emerging. For example, Alves et al., present data describing testosterone's ability to control vascular dysfunction in an inflammasome (NLRP3)-dependent manner, and Wilhelmson et al. present new data supporting a role for thymic epithelial cells in driving androgen-dependent early T cell development and the accumulation of recent thymic emigrant T cell populations in spleens and lymph nodes. Finally, Tuku et al. present data suggesting that the primary cytokine response to H1N1-driven lung infection is guided by androgens, driving sex differences observed among individuals infected with this strain of influenza. Thus, testosterone likely also affects key target non-immune cells in tissues susceptible to (auto)immune regulation, thus further amplifying the differential development of autoimmunity, cancer and infectious disease responses between men and women (reviewed by Ben-Batalla et al.).

As the medical field is driven toward personalized medicine and the availability of sex-specific genetic and biological data and tests rise in numbers, there is increasingly a need, but also the possibility, for identification of endogenous variability between individuals. Hundreds of genes coding for immune-related proteins differ between individuals, providing insight into the complexity of personalized medicine (4). Thus, it may not be surprising that our ability to mount and control immune responses has repeatedly been found to also differ between the sexes. As a consequence, different populations are known to be disproportionately affected by autoimmunity and cancer [reviewed in (5)], with the sex of individuals representing a significant variable.

In summary, further studies are needed in controlled environments for us to fully understand the complex regulation imposed by sex hormones. Still, with the knowledge on hand, the development of small-molecule agonists and antagonists capable of affecting selected downstream sex-dependent regulatory pathways, or target sex hormone receptors in selected cells or tissues, is of great importance for the field as we move toward the development of *de novo* therapeutic agents capable of lowering the severity of diseases presenting with a sex-bias.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this publication. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Testosterone Protects Against Severe Influenza by Reducing the Pro-Inflammatory Cytokine Response in the Murine Lung

Berfin Tuku[1†], Stephanie Stanelle-Bertram[1†], Julie Sellau[2], Sebastian Beck[1], Tian Bai[1], Nancy Mounogou Kouassi[1], Annette Preuß[1], Stefan Hoenow[2], Thomas Renné[3], Hanna Lotter[2] and Gülsah Gabriel[1,4]*

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Influenza A virus pathogenesis may differ between men and women. The 2009 H1N1 influenza pandemic resulted in more documented hospitalizations in women compared to men. In this study, we analyzed the impact of male sex hormones on pandemic 2009 H1N1 influenza A virus disease outcome. In a murine infection model, we could mimic the clinical findings with female mice undergoing severe and even fatal 2009 H1N1 influenza compared to male mice. Treatment of female mice with testosterone could rescue the majority of mice from lethal influenza. Improved disease outcome in testosterone treated female mice upon 2009 H1N1 influenza A virus infection did not affect virus titers in the lung compared to carrier-treated females. However, reduction in IL-1β cytokine expression levels strongly correlated with reduced lung damage and improved influenza disease outcome in female mice upon testosterone treatment. In contrast, influenza disease outcome was not affected between castrated male mice and non-castrated controls. Here, influenza infection resulted in reduction of testosterone expression in male mice. These findings show that testosterone has protective functions on the influenza infection course. However, 2009 H1N1 influenza viruses seem to have evolved yet unknown mechanisms to reduce testosterone expression in males. These data will support future antiviral strategies to treat influenza taking sex-dependent immunopathologies into consideration.

Keywords: influenza A virus, sex differences, testosterone, 2009 H1N1, androgens

INTRODUCTION

Females in their reproductive age experience more severe disease following influenza A virus infection than males (1, 2). Hospitalization rates during influenza seasons were reported to be higher in male children and in elderly males. However, during the reproductive age, females are more likely to be hospitalized than males (3). This was further supported and highlighted during the 2009 H1N1 influenza pandemic. Here, females were reported to develop more severe influenza disease compared to males in multiple published datasets (4–6). In line, females were more likely

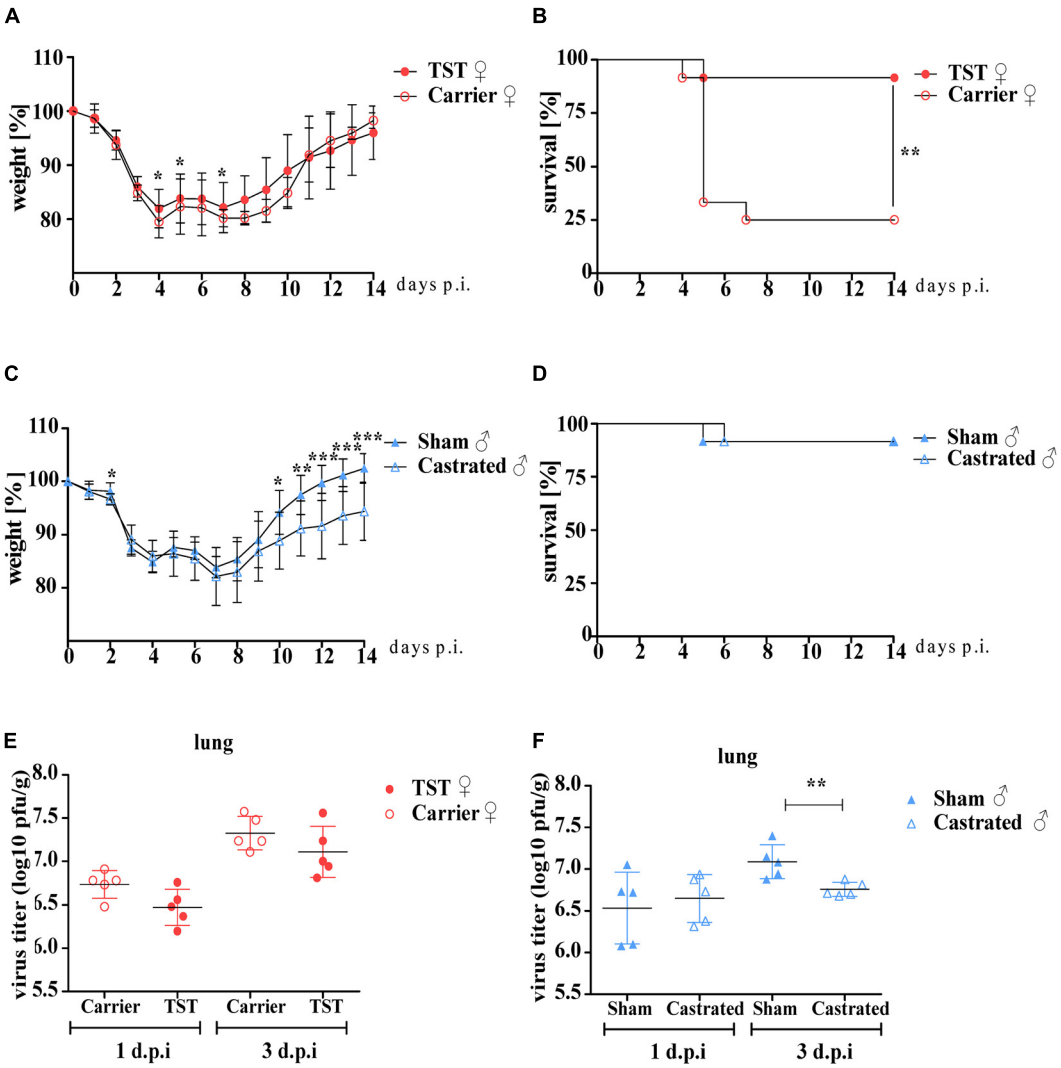

FIGURE 1 | Testosterone impact on 2009 H1N1 influenza A virus pathogenicity in female and male C57BL/6 mice. Male mice (n = 12 each) were gonadectomized or sham-operated. Female mice (n = 12 each) were implanted an osmotic pump releasing either testosterone (TST) or a carrier substance. Female and male mice were intranasally infected with 1×10^4 of the 2009 H1N1 influenza A virus. Weight loss and survival **(A–D)** were monitored for 14 days. Mean values and SD were determined. Statistical significance was assessed by Mantel–Cox test for the survival data and Student's t-test for the weight loss data (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Lungs of five animals per group were harvested on days 1 and 3 d.p.i. Viral lung titers were determined by plaque assay **(E,F)**. The individual logarithmic virus titers of each lung and their means are shown. Statistical significance was assessed by Student's t-test (*$p < 0.05$).

to die upon avian H7N9 or H5N1 influenza A virus infection than males (7, 8). These epidemiological observations could be mimicked in murine infection models (7). Yet, the underlying mechanisms of sex-specific influenza disease outcome are largely unknown. In males, testosterone is the predominant sex hormone (further described as "male sex hormone"), whereas in females the predominant sex hormones are estradiol and progesterone (further described as "female sex hormones"). In a murine influenza infection model, it was shown that the female sex hormones, 17β-estradiol and progesterone, have a protective effect on influenza disease outcome in females (9, 10). In this study, we analyzed the impact of the major male sex hormone testosterone on influenza disease outcome using a pre-clinical murine infection model.

MATERIALS AND METHODS

Animal Experiments

C57Bl/6JRccHsd mice were purchased from Envigo RMS Harlan Laboratories (Rossdorf, Germany). Eight weeks old mice were anesthetized intraperitoneally with ketamine-xylazine (100 and 10 mg/kg, respectively) and intranasally infected with 10^4 p.f.u. 2009 H1N1 (A/Hamburg/NY1580/09) influenza virus (11) diluted in 50 µl 1x phosphate buffered saline (PBS). Control

groups received 50 µl 1x PBS. Animal numbers are provided in the legends. After infection, body weight and mortality were monitored for 14 days. At selected time points, whole lungs and plasma were collected for terminal studies.

Surgical Procedures, Testosterone Administration, and Quantification

Male and female mice were anesthetized with 2.5% isoflurane mixed with oxygen. For gonadectomy, 6 weeks old male mice were assigned to remain intact or be bilaterally gonadectomized. Six weeks old female mice were subcutaneously implanted an ALZet Model 2004 micro-osmotic pump (Charles River) releasing either a carrier substance for the control group or testosterone [5 mg/ml diluted in 45% w/w (2-Hydroxypropyl)-ß-cyclodextrin]. One hour before and 24 h after surgery mice were administered subcutaneously with carprofen purchased from Zoetis (5 mg/kg) as postoperative analgetic. All infections occurred 2 weeks following the surgeries. Plasma testosterone concentrations were determined by a chemiluminescence immunoassay (ADVIA Centaur Testosterone II assays; Siemens Healthcare Diagnostics) and the measurements were performed with the ADVIA Centaur XP (Siemens Healthcare Diagnostics).

Lung Pathology

Formalin-fixed paraffin-embedded lung thin sections were stained with hematoxylin a rabbit anti-NP antibody (Thermo Fisher, PA5-32242) and a biotin-conjugated-rabbit secondary antibody (Jackson ImmunoResearch, 711-066-152) for immunohistochemical analysis. All images were taken at 400x magnification with a wide-field microscope (Nikon Eclipse 80i live microscope).

Pulmonary Cytokine/Chemokine Quantification and Analysis of Viral Lung Titers

Homogenization of ∼ 50 mg of lungs was performed in 1 ml PBS with 5 sterile, stainless steel beads (Ø 2 mm, #22.455.0010, Retch) at 30 Hz and 4°C for 10 min in the mixer mill MM400 (Retsch). Viral lung titers were determined by plaque assay as described previously (7). Cytokine and chemokine levels were analyzed using ProcartaPlex™ multiplex immunoassays (Thermo Fisher) according to the manufacturer's protocol. The following cytokines and chemokines were analyzed: interleukins (IL): IL-1β, IL-6, IL-10, IL-17A, interferon (IFN) α, tumor necrosis factor (TNF) α, and monocyte chemotactic protein (MCP) 1. The signal intensities were measured using Bio-Plex® 200 Systems (Bio-Rad).

Analysis of Sex Hormone Receptor Level Expression by RT-qPCR

Total RNA from PBMCs was isolated following a guanidinium thiocyanate-phenol-chloroform extraction protocol. The samples were diluted in TRIzol® and treated with chloroform before centrifugation. After phase-separation, the precipitation of RNA was performed using isopropanol following a washing step with 75% Ethanol. The RNA was eluted in RNase-free water. RNA concentration and purity were determined using the Nanodrop 1000 (Peqlab). Total cDNA was generated using random nonamer primers (Gene Link™, pd(N)9, 26-4000-06) and the SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. For the qPCR, specific primer pairs were used for the genes of interest (GOIs), murine estrogen receptor alpha (ESR1), murine androgen receptor (AR) and for the reference gene murine ribosomal protein 9 (Rsp9). The reactions were set up in MicroAmp® Optical 96-Well Reaction Plates (Invitrogen) including Platinum® SYBR® Green qPCR SuperMix-UDG (ROCHE®), forward and reverse primer and the cDNA template. The RT-qPCRs were conducted on the LightCycler® 96 Real-Time PCR System (ROCHE®) as described previously (12). The relative quantifications were performed using the $-2^{-\Delta\Delta Ct}$ method.

The following primer sequences were used for qRT–PCR:
murine ESR1 forward 5′-AGTGAAGCCTCAATGATGGG-3′,
reverse 5′-GAGCAAGTTAGGAGCAAACAG-3′,
murine AR forward 5′- TGAGTACCGCATGCACAAGT-3′,
reverse 5′- GCCCATCCACTGGAATAATGC-3′

Data Analysis

All data were analyzed with the Prism software (GraphPad, 5.03) using Mantel–Cox test or Student's *t*-test as indicated in the respective legends. Association between sex hormones and cytokines were determined using linear regression and correlation analysis (Pearson). Statistical significance was defined as $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

Testosterone Treatment Protects Female Mice From Lethal 2009 H1N1 Influenza A Virus Infection

Influenza A virus pathogenesis may vary depending on sex (2, 7). Here, we studied the impact of testosterone on influenza disease outcome in female and male mice.

Female mice were either implanted with a testosterone releasing osmotic pump or with a carrier substance releasing pump as a negative control. Two weeks after surgery, testosterone and carrier treated female mice were infected with 2009 H1N1 influenza A virus (pH1N1). Testosterone treated females underwent reduced weight loss compared to carrier treated females (**Figure 1A**). While 2009 H1N1 infection was highly lethal (75%) in carrier treated females, testosterone treated females displayed high survival rates (92%) (**Figure 1B**).

Male mice were castrated or sham-operated to study the impact of testosterone on influenza disease outcome. Castrated male mice underwent more weight loss during the recovery phase compared to sham-operated control males upon 2009 H1N1 infection (**Figure 1C**). However, survival rates did not differ between infected castrated and non-castrated males (**Figure 1D**). Even increasing 2009 H1N1 infection dose did not significantly affect weight loss or survival rates (**Supplementary Figure S1**).

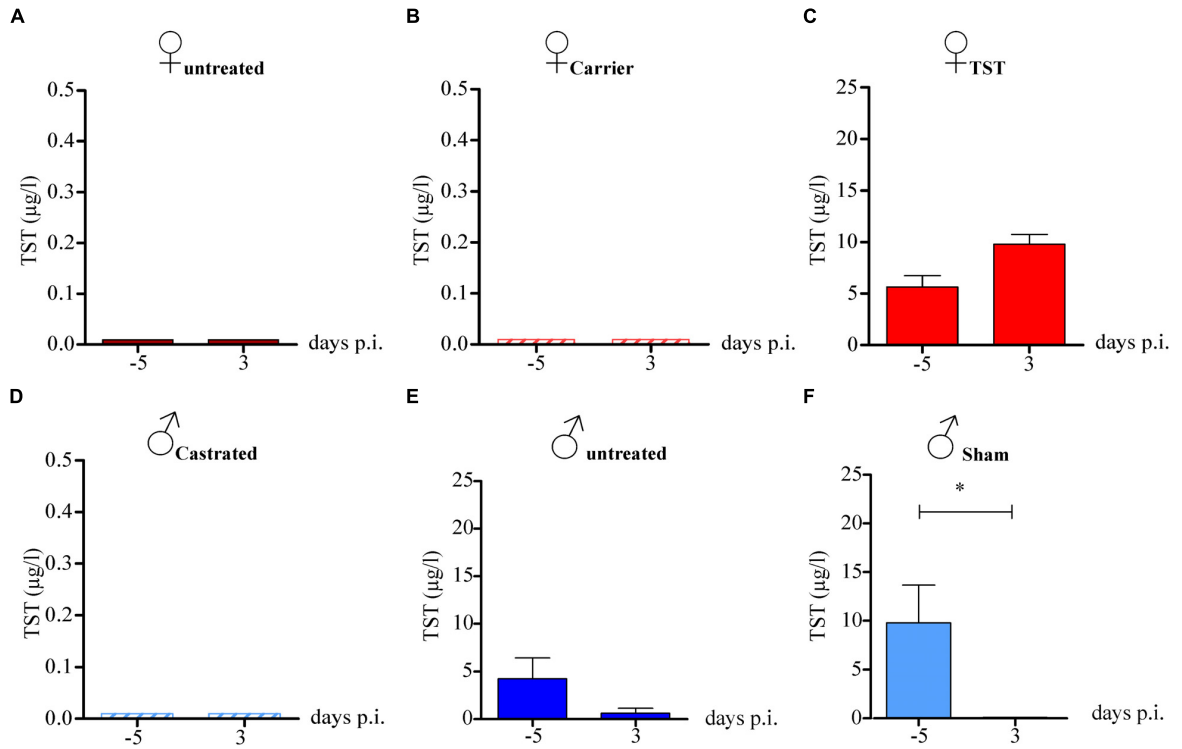

FIGURE 2 | Testosterone levels in 2009 H1N1 influenza A virus infected female and male mice. Non-treated female mice **(A)**, female mice with an implanted osmotic pump releasing either a carrier substance **(B)** or testosterone (TST) **(C)** as well as gonadectomized **(D)** (n = 5 each), non-treated **(E)** and sham-operated male mice **(F)** (n = 10 each) were intranasally infected with 1×10^4 of the 2009 H1N1 influenza A virus. Serum testosterone levels were measured on day 5 before infection and day 3 post infection. Statistical significance was assessed by Student's t-test (*$p < 0.05$).

Virus replication titers in the lungs of testosterone treated female mice were comparable to carrier treated control females, albeit a tendency toward lower replication upon testosterone treatment could be detected at both 1 day and 3 days post infection (p.i.) (**Figure 1E**). Virus replication did not differ between castrated and sham-operated male mice on day 1 p.i. On day 3 p.i., virus replication was reduced in the lungs of castrated compared to control males (**Figure 1F**).

These findings show that female mice treated with testosterone are protected against lethal 2009 H1N1 influenza. However, improved survival rates do not correlate with reduced virus lung titers suggesting that the underlying mechanism is not primarily dependent on virus replication. Moreover, the protective role of testosterone is not observed in male mice.

Testosterone Expression Levels Are Reduced in 2009 H1N1 Influenza A Virus Infected Male Mice

Next, we wanted to assess whether 2009 H1N1 infection might affect testosterone levels that in turn could affect disease outcome. Therefore, we measured testosterone levels in the plasma of mice 5 days (−5) before and 3 days (3) p.i. In female mice, either non-treated or treated with carrier substance, testosterone levels were below detection limits as expected (**Figures 2A,B**). Females treated with testosterone displayed testosterone levels within the physiological range of male mice before and during infection (**Figure 2C**). Castrated male mice did not show detectable testosterone levels as expected (**Figure 2D**). Male mice, either sham-operated or non-treated displayed high testosterone levels before infection. However, testosterone levels were significantly reduced in non-treated and sham-operated males on day 3 p.i. (**Figures 2E,F**). Then, we assessed whether changes in hormone levels might be due to altered hormone receptor expression. No significant differences in estrogen receptor (ESR1) and AR expression were detected in infected versus non-infected groups. However, a slight decrease ESR1 expression could be observed in testosterone treated female mice irrespective of infection (**Supplementary Figure S2**).

These findings show that 2009 H1N1 infection mediates reduced expression of testosterone in male mice. This observation may explain why influenza virus pathogenesis did not differ between castrated and non-castrated males.

Testosterone Treatment Dampens Inflammatory IL-1β Response in 2009 H1N1 Influenza A Virus Infected Female Mice

Then, we addressed the question whether testosterone might affect pulmonary chemokine and cytokine responses in 2009

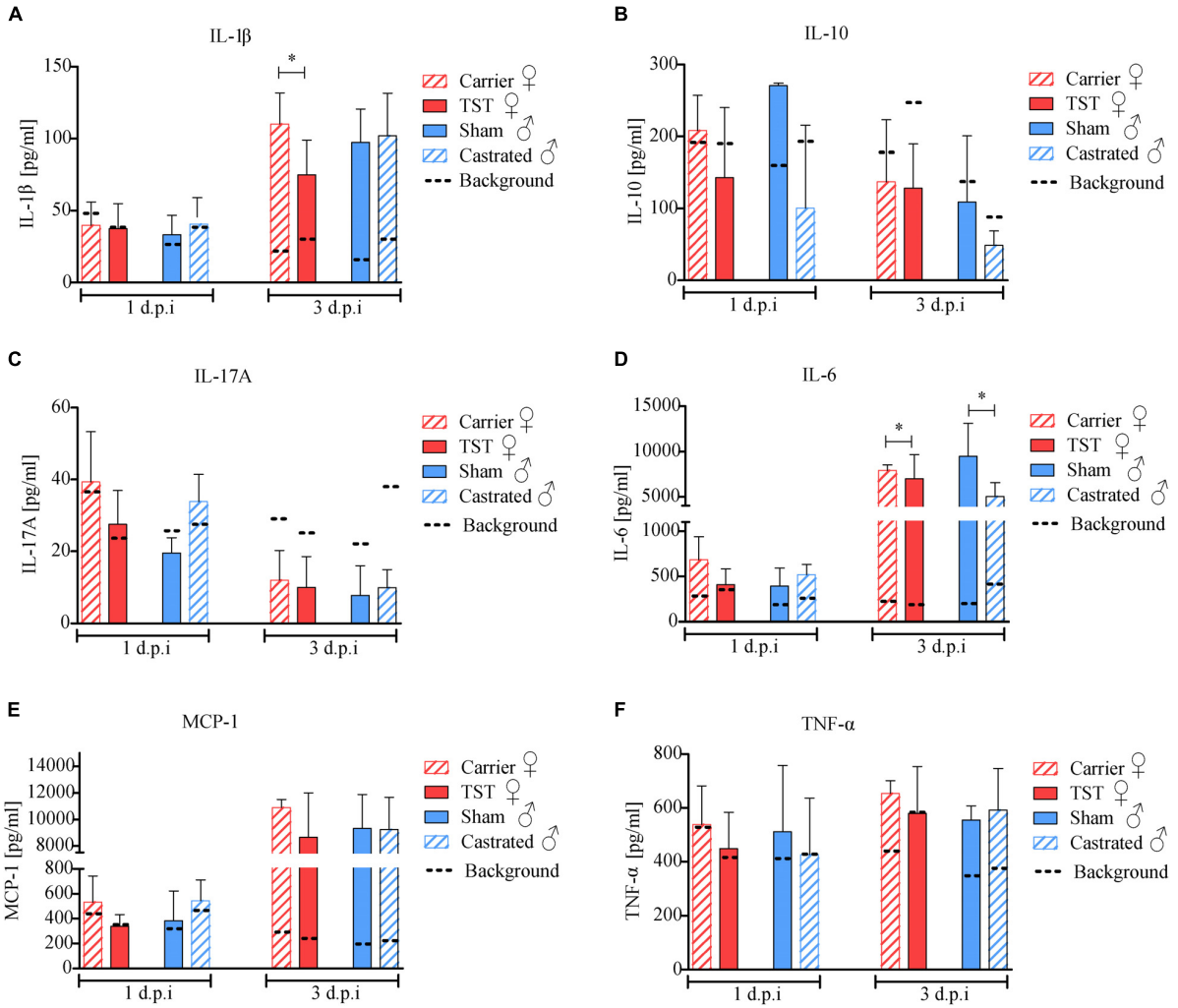

FIGURE 3 | Testosterone impact on chemokine and cytokine responses in 2009 H1N1 influenza A virus infected female and male C57BL/6 mice. Gonadectomized or sham-operated male mice (n = 5 each) and female mice with an implanted osmotic pump releasing either testosterone (TST) or a carrier substance (n = 5 each) were intranasally infected with 1×10^4 of the 2009 H1N1 influenza A virus or PBS. Lungs of five animals per group were harvested on days 1 and 3 d.p.i. Cytokines IL-1β **(A)**, IL-10 **(B)**, IL-17A **(C)**, IL-6 **(D)**, MCP-1 **(E)**, TNF-α **(F)** were determined in lung homogenate supernatants (n = 5) by a procartaplex cytokine multiplex assay. The individual mean values of the PBS control animals are indicated as dotted lines. Statistical significance was assessed by Student's t-test (*$p < 0.05$).

H1N1 infected mice thereby affecting disease outcome. As a control, cytokine and chemokine levels of PBS-treated mice were defined as background references and compared individually to the respective treated groups. Upon 2009 H1N1 infection, IL-1β, IL-6, and MCP-1 level were increased in all animals compared to their respective background references unlike IL-10, IL-17A, and TNF-α levels (**Figures 3A–F**). Testosterone treated female mice showed significantly decreased IL-1β levels compared to carrier substance treated females (**Figure 3A**). IL-6 level were slightly decreased in testosterone treated females and in castrated males (**Figure 3D**). However, cytokine and chemokine levels were not significantly altered in the respective male groups in line with similar disease outcomes.

These data show that testosterone treatment results in significantly reduced pro-inflammatory IL-1β expression in the lungs of 2009 H1N1 infected female mice correlating with elevated survival rates in females.

Testosterone Reduces Lung Pathology in 2009 H1N1 Influenza A Virus Infected Mice

We then assessed the impact of testosterone treatment on lung pathology in 2009 H1N1 infected female and male mice. 2009 H1N1 infection resulted in antigen-positive bronchial as well as alveolar epithelium in female and male mice (**Figure 4**). Female mice treated with testosterone displayed reduced infiltration with mononuclear cells accompanied by less alveolar destruction compared to lungs of carrier-treated mice. Sham-operated and castrated male mice showed similar infiltration and virus positive cells.

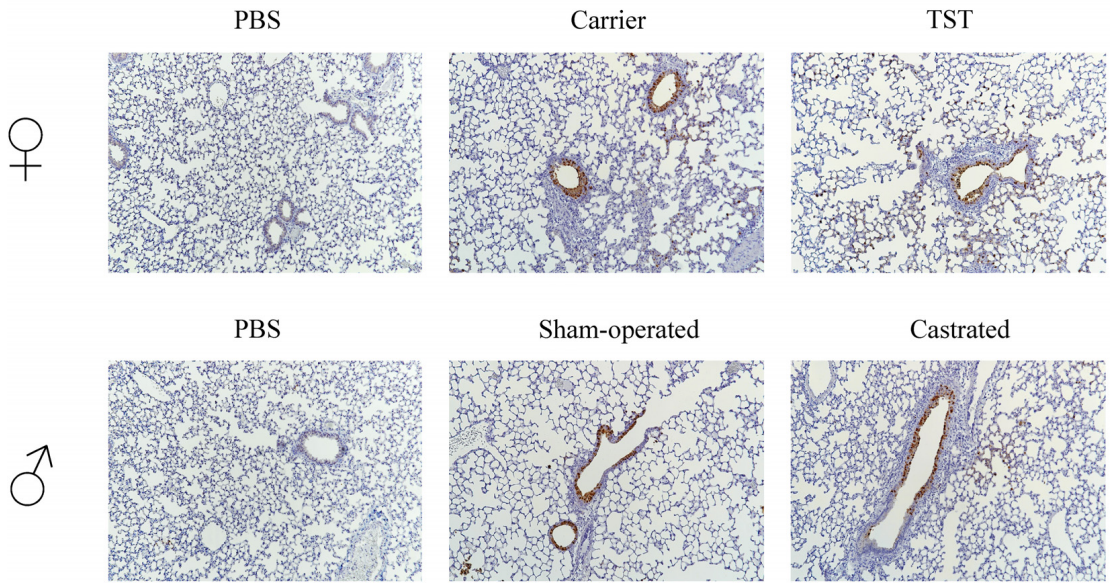

FIGURE 4 | Testosterone impact on lung tropism of 2009 H1N1 influenza A virus infected mice. Female mice with an implanted osmotic pump releasing either a carrier substance or testosterone (TST), sham-operated or gonadectomized male mice (n = 5 each) were either intranasally infected with 1×10^4 of the 2009 H1N1 influenza A virus or inoculated with PBS as a control. On day 3 p.i., lungs were harvested for histopathological analysis. Stainings were performed against influenza A virus nucleoprotein.

These data show that testosterone treatment reduces lung pathology in female mice correlating with ameliorated influenza disease outcome.

DISCUSSION

In this study, we sought evidence for the epidemiological observation why men undergo less severe influenza than women. These clinical findings can be mimicked in murine infection models as shown earlier allowing now causal assessments (1, 7). In this study, we provide evidence that testosterone has a protective role in 2009 H1N1 disease outcome in females. Testosterone is known to have an anti-inflammatory impact (13), whereas estrogens promote inflammation (14, 15). Severe inflammation is associated with cytokine storm leading to severe influenza (16). However, this protective impact of testosterone is not observed in male mice. Detailed analysis of testosterone expression kinetics in male mice revealed that 2009 H1N1 influenza virus infection reduces testosterone expression levels in male mice. This phenomenon is not expressed in female mice likely due the generally low testosterone levels in females. Others have reported a decline of testosterone levels in young and elderly male mice before (10). However, the underlying mechanism how influenza A virus infection might dysregulate testosterone expression is unknown. It was reported before that cytokines might interfere with testosterone synthesis, albeit the detailed mechanism is still unclear (17). In this study, we further analyzed androgen and receptor expression levels to identify the potential target pathways. However, influenza A virus infection did not significantly alter androgen or ESR1 levels, respectively (**Supplementary Figure S2**). Thus, future studies are required to understand the viral interference with testosterone expression. However, we could identify that testosterone treatment of female mice improves virus induced lung damage without affecting respiratory virus replication kinetics. Improved disease outcome in influenza virus infected female mice upon testosterone treatment strongly correlated with reduced pro-inflammatory IL-1β cytokine levels in the lungs and high survival rates. IL-1β plays a key role in influenza virus mediated lung pathologies (18). Interestingly, it was shown before that aged male mice treated with testosterone had reduced pulmonary IL-1β levels compared to aged male mice with low testosterone levels after infection (19). Therefore, a potential correlation between testosterone and IL-1β serum level in female mice after infection has been analyzed (**Supplementary Figure S3**). The results indicate a trend for a negative correlation between testosterone levels and IL-1β levels. In summary, our data show that testosterone treatment of females significantly improves influenza disease outcome by dampening IL-1β responses and reducing virus induced lung damage. These findings highlight potential sex differences should be taken into consideration in developing new antiviral strategies against pandemic influenza.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the German authorities (Behörde für Gesundheit und Verbraucherschutz, Hamburg, Germany, license number 01/15) and conducted according to the FELASA guidelines of animal welfare. All animal experiments were performed according to the guidelines of the German Animal Welfare Regulation.

AUTHOR CONTRIBUTIONS

BT, SS-B, and GG designed the study. BT and SS-B performed the experiments, analyzed the data, and performed all animal infection experiments. BT and GG wrote the manuscript. JS, SH, and HL performed the mouse surgeries. SB, AP, and TB supported animal infection experiments. NK performed the lung histopathological analysis. TR measured and analyzed the testosterone levels in mice. All authors revised the manuscript.

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Androgen-Mediated Anti-inflammatory Cellular Processes as Therapeutic Targets in Lupus

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Systemic Lupus Erythematosus (SLE), among many other auto-immune diseases, is known to be more prevalent in females than in males. This observation has served as the foundation for studies into how sex hormones may interact with the immune system to either drive or inhibit immune activation. Early studies using castration in lupus mouse models showed the potential protective effect of testosterone against lupus development. These studies were later corroborated by observational studies in lupus patients, who upon treatment with testosterone therapy, displayed decreased disease burden. However, there are numerous limitations to treating (especially female) lupus patients with testosterone. Thus, identification of testosterone-targeted cellular and molecular mechanisms affecting immune activation is an attractive target for lupus treatment in the future. Recent studies have examined the effects of androgens on the activation of anti-inflammatory processes. As such, immunoregulatory cell types including myeloid-derived suppressor cells (MDSCs) and regulatory T and B cells have been shown to be susceptible to manipulation by sex hormones. Here, we review studies of SLE and lupus-like disease in which testosterone or testosterone-derivatives were used to skew an ongoing immune reaction toward an anti-inflammatory state. Via evaluation of both clinical studies and immunologic models we propose new areas for research with the goal of identifying testosterone-driven anti-inflammatory mediators suitable for therapeutic targeting in patients with lupus and other autoimmune diseases.

Keywords: SLE, lupus, androgen, MDSC, pDC

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INTRODUCTION

SLE is an autoimmune disorder which may target multiple organs, including skin, joints, and kidneys. It has an annual incidence of 7.2 per 100,000 individuals a year in the United States (1). Current standard disease management involves the use of glucocorticoids initially, with added maintenance immunosuppressive therapy as needed. These options, however, are broadly immunosuppressive, have significant adverse effects, and do not directly target the cause of lupus.

The pathogenesis of SLE is multi-factorial with genetic as well as environmental factors implicated. Although men with lupus have been shown to have a worse course of disease than women, women are much more predisposed to getting lupus than men, with a 9:1 female to male ratio among patients. Interestingly, this ratio is the highest during reproductive years, when sex hormone levels are the highest. Moreover, disease incidence peaks in women during their

reproductive years (30–50 years), while in men there is a later peak at ages 50–80 years (1). This female predisposition is poorly understood in its relation to the pathogenesis of lupus, but suggests that sex hormones play a role in disease initiation or progression. We and others have previously reviewed how sex hormones interact with the immune system in a variety of ways [reviewed in (2, 3)], however our understanding of their specific role in lupus pathogenesis remains limited. Thus, a better understanding of the relationship between sex hormones, cellular and molecular targets of sex hormones, and lupus pathogenesis may lead to new targeted therapies. In this review we will focus on clinical and laboratory studies evaluating the role of androgens in SLE and mouse lupus-like disease (lupus); highlighting potential areas of further study to improve SLE therapies, particularly within testosterone's role as an immune regulator.

ANDROGEN LEVELS AND MANIPULATIONS IN SLE

Early studies of sex hormone levels in SLE patients showed that female patients with active disease expressed decreased levels of androgens, including testosterone, androstenedione, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) (4, 5). Decreased levels persisted even after standard of care treatment and the induction of remission (6), suggesting that low levels were not a result of disease, but intrinsic to the patient population. Additional studies of testosterone therapy in SLE patients suggested a benefit from androgen therapy to disease severity (7–9), however a subsequent study failed to show improved disease activity, quality of life or sexual functioning in women with mild/moderate lupus (10). More recently, two larger studies using DHEA at a dose of 200 mg in women with SLE found a higher percentage of patients showing stable or improved disease within the DHEA group compared to the placebo group (11, 12). While reasons for the discrepancy in these results remain unclear, it should be noted, that steroid use has been identified as a potential confounder when assessing the efficacy of testosterone (13, 14). Thus, further analyses evaluating the clinical efficacy of testosterone and DHEA in SLE patients as a function of steroid use are needed to establish whether such treatments should be more widely offered.

In men with SLE, androgen levels have also been shown to be reduced (15–18), and while an early study showed no evidence of hypogonadism or androgen deficiency (15), a more recent study showed that hypoandrogenism was present in a subset of males with lupus (16). Circumstantially, treatment of male SLE patients with 19-norandrostione resulted in increased serum estrogen levels—and reduced testosterone levels—and elevated serum anti-dsDNA antibodies (18), supporting a correlation between low androgen levels and elevated autoantibodies. Interestingly, a separate study comparing testosterone levels in different patient groups with other chronic diseases also showed reduced levels across all study participants, raising the possibility that low levels of androgens are a result of the chronic inflammatory milieu in all patients and not just in lupus (17).

Finally, sex hormones are altered among transgender patient on hormone therapies. A few case reports exist of male-to-female transgender patients who developed lupus following the use of feminizing sex hormones (19–21), and a single case report of a female-to-male transgender patient, reported an established subacute cutaneous lupus erythematous prior to hormone therapy with resolution of symptoms following androgen replacement therapy (ART) (22). To our knowledge no reports have been published supporting resolution in male-to-female patients or lupus development in female-to-male patients, further supporting a protective effect of androgens and an exacerbating effect of estrogens. In summary, most studies suggest a possible therapeutic role for androgens themselves, although unwanted side effects have prevented further development of such treatments. Further identification of specific cellular and/or molecular targets of testosterone within the immune system may however represent a promising area for development of future SLE therapies.

ANDROGEN MANIPULATION IN ANIMAL MODELS OF LUPUS

Given the observations about sex hormones and lupus disease progression in SLE patients, hormone manipulation studies in animal models of lupus have been carried out, primarily using NZB/NZW F1 mice, which have a female predominance for SLE-like disease. These studies, showed a protective role for testosterone in mouse lupus (and a disease promoting role for estrogens), providing a model system in which the cellular targets and mechanisms of action of testosterone can be further studied [reviewed in (3)]. As research with this and other animal models has continued, a number of androgen mediated pathways have emerged as potential future therapy targets, particularly among regulatory cell populations and their activation by androgens.

REGULATORY CELLS AS TARGETS OF ANDROGENS?

Regulatory T Cells (Treg)

Regulatory T cells are represented by the natural thymic Tregs and a population of induced Tregs (iTreg) (23). Support for an association between testosterone and Tregs recently came from a study of children, in which it was found that 8 years old boys with higher levels of cord blood DHT levels at birth expressed increased levels of Tregs as compared with boys with lower levels of cord blood DHT and girls (24). In animal models, however, the literature discussing direct effects of androgens on Tregs is sparse and inconclusive. For example, on one hand it has been shown that testosterone directly affects the expression level of Foxp3 via an androgen-receptor binding motif in the proximal *Foxp3* promoter (25), while on the other hand, gonadectomy of male mice in models of virus-induced myocarditis and autoimmune hepatitis resulted in increased or no changes in Tregs, respectively (26, 27). In SLE, reduced levels and functions of Tregs have been reported in two independent studies (28, 29), while a third study surprisingly showed elevated levels of

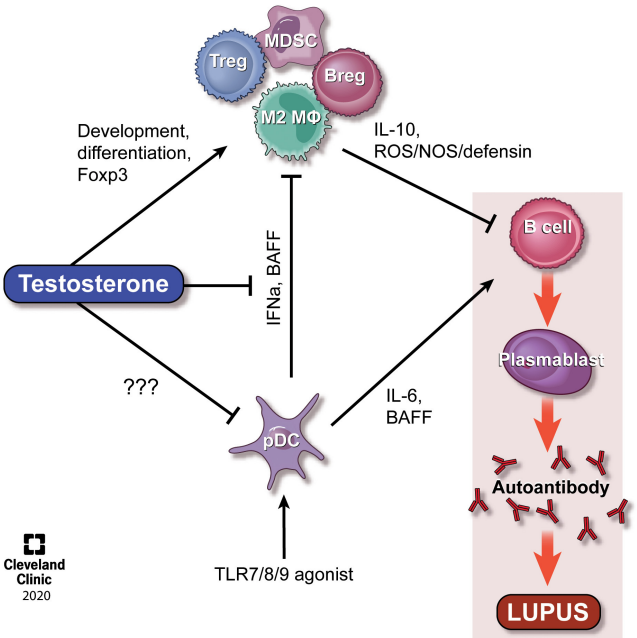

FIGURE 1 | Model of the effect of testosterone on regulatory cells and the opposing effects of pDCs. It is well-established that TLR-stimulated pDCs secrete IFNα, IL-6, and BAFF, all of which actst to promote immune activation and lupus pathogenesis. Testosterone exert direct effects on the development of MDSCs and Tregs, the latter via regulation of Foxp3, and indirect effects on M2 macrophages and Bregs, potentially via regulation of BAFF. The balance between testosterone and pDC/IFNα levels represent an interesting area for therapeutic targeting in SLE. Please see the text for additional details. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2020. All Rights Reserved.

Tregs (30). Interestingly, the latter study also showed that IFNα production from SLE-derived antigen-presenting cells (APCs), but not from healthy control APCs, was responsible for inhibiting Treg functionality (30), suggesting that Treg abnormalities may be a result of elevated IFNα levels and the chronic inflammatory environment of SLE patients (see **Figure 1**). Therapeutically, adoptive transfer of Tregs in lupus has been investigated. A single lupus patient was treated with autologous Tregs, and the treatment resulted in increased Tregs at cutaneous inflammatory sites, as well as a shift from a Th1 to Th17 response (31). While results from only one patient are difficult to draw any conclusions from, it does support a role for investigating Tregs within the pathogenesis of lupus.

Regulatory B Cells (Breg)

B cells are known to have a number of actions within the pathogenesis of lupus, most notably the production of autoantibodies. However, a subset of B cells known as Bregs play a suppressive role, mainly through the actions of IL-10 and TGF-β and have recently emerged as a focus within lupus (32). In healthy individuals, Bregs have been found to suppress the differentiation of Th1 cells following CD40 stimulation in an IL-10-dependent manner (33). Although Bregs have been found at increased levels in patients with SLE (34), it has also been reported that SLE Bregs are unable to suppress Th1 differentation, and have decreased capacity to produce IL-10 when stimulated with CD40 (33) and TLR9 (35). Interestingly, this dysregulation of Bregs in lupus may be driven through IFNα produced by pDCs, promoting plasmablast differentiation while suppressing Breg differentiation (36) (see **Figure 1**). Of note, recent drug trials in lupus included testing of compound BT063, a monoclonal humanized anti-IL-10 antibody; tested for safety and tolerability. The study met its primary endpoint for safety and tolerability, and additionally showed early signs of efficacy (35). This choice of target is interesting, given the immunosuppressive actions of IL-10, and it remains to be seen whether further studies of this drug will indeed show efficacy, or if a different target within the IL-10 activation pathway may prove to be more appropriate.

While there are no studies, to our knowledge, supporting direct effects of androgens on the development of Bregs, testosterone is known to suppress B cell expansion in general (37, 38), and may hence suppress Bregs as well. Alternatively, testosterone may drive Breg differentiation indirectly via effects of other cells involved in the differentiation and/or maintenance of Bregs. For example, a study by Olsen found that bone marrow stromal cells were required, and mediated the B cell suppressive effects of androgens through TGF-β secretion (39). Thus, in this case androgens exerted their effect primarily on pro-B cell populations centrally, with little effect on peripheral cells, potentially redirecting differentiation of B cells toward a more suppressive phenotype as well. Testosterone have also been found to directly regulate BAFF levels, a key mediator of B cell development and maintenance (40). Using a scleroderma model, Matsushita et al. found that BAFF suppressed Bregs, while a BAFF antagonist reduced B effector cells but did not significantly alter Bregs (41), thus providing a molecular mechanism for a skewing toward Bregs in males. Further studies are needed to elucidate if other mechanisms also facilitate a role for testosterone in B cell maturation. Belimumab, an antibody against BAFF, is currently FDA approved for lupus and has been shown to have some effect in SLE patients, altering multiple B cell subsets (42). While data specifically on Breg levels before/after treatment have not been reported, there appears to be alterations in expression of some of the markers for Bregs (42). A number of other anti-BLys and anti-APRIL drugs have been studied (Atacicept, Blisibimod, Tabalumab), but have not seen the same level of success (43, 44). There is an ongoing study to investigate the pathways in which BAFF and APRIL act in order to identify specific patient populations that would be ideal candidates for belimumab among lupus patients (NCT03919643). It would be interesting to see if this trial finds any differences in Breg levels and if gender or hormone levels associate with response to therapy.

M2 Macrophages

Macrophages have diverse phenotypes and can be distinguished as inflammatory M1 macrophages and anti-inflammatory/repair M2 macrophages (45). While M1 macrophages are known to be pathogenic in lupus, recent studies have suggested a role for M2 macrophages in damping the immune response (46, 47). As such, adoptive transfer of M2 resulted in a reduction in SLE severity in a mouse model (48). Therapeutically, PAM3, a TLR2/1 agonist,

has been shown to skew macrophages toward a M2 phenotype in lupus mouse models (49), however the use of PAM3 as a therapeutic agent is controversial and may likely spike unwanted immune activation.

Testosterone acts on macrophages via androgen receptors (50, 51), however few studies have evaluated testosterones effect specifically on M2 macrophages. In one example, Ma et al. found that blocking the androgen receptor alleviated inflammation and promoted M2 polarization in a myocarditis *in-vitro* model (52). Similarly, a recent study by Zhu found that blocking the AR in renal cells led to decreased kidney stones and increased M2 levels (53), suggesting that androgen receptors exert proinflammatory functions in some disease settings.

Oppositely, among alveolar macrophages (AM) from asthma patients, dihydrotestosterone reduced lung inflammation, and enhanced M2 polarization of AM, despite the finding that M2 macrophages were previously found to correlate with asthma severity (54). While the phenotype of M2s within the alveolar space may not be representative to M2s involved in autoimmune pathologies, the finding suggests that among a more heterogeneous population of M2s, androgens may play a specific immunosuppressive stimulatory role. Finally, as mentioned above, testosterone reduces BAFF secretion (40) and when the BAFF antagonist BAFF-Trap was used in a rheumatoid arthritis model, animals exhibited reduced numbers of DCs but increased levels of not only Tregs and Bregs, but also M2 macrophages (55). Thus, BAFF, as a key regulator of lupus pathogenesis may affect multiple regulatory pathways, and be regulated in part by testosterone. In summary, the role of androgens in M2 activation/suppression remains unclear and more research is needed to unravel both direct and indirect effects.

Myeloid-Derived Suppressor Cells (MDSCs)

Myeloid derived suppressor cells are a heterogeneous population of immature myeloid cells, which have been shown to suppress immune functions during inflammatory conditions. The cells have been shown to have deleterious effects on cancer [reviewed in (56)], but may hold therapeutic potential in suppressing autoimmune disorders. However, results on their function in autoimmunity have been conflicting, owing likely to the diverse cell subpopulations included within the population of MDSCs (57).

Using the NZB/NZW F1 mouse model of lupus, we found previously that MDSCs are significantly increased in male mice in a testosterone dependent fashion (58). Moreover, *in vitro* studies showed that MDSCs were functionally suppressive in post-pubertal male, but not female, mice, and that *in vivo* depletion of these cells resulted in elevated autoantibody production in males, but not females (58). Further analyses showed that $Gr1^+$ cell-depleted male NZB/NZW F1 mice displayed expanded populations of splenic germinal center B cells, plasma cells and T follicular helper cells, critical for driving an antibody response, as well as significantly elevated levels of IL-10 (58, 59). Subsequent studies by Bird et al. in female NZB/NZW F1 mice corroborated these studies and showed that early depletion of MDSCs accelerated disease development, while later depletion had no effect (60). Also, a study of the Sanroque mouse model of lupus displayed a similar phenotype with MDSCs inhibiting germinal center B cells, plasma cells and T follicular helper cells, while promoting IL-10-producing B cells (61) (see **Figure 1**).

MDSCs have been found to be subject to differentiation to pro-inflammatory dendritic cells or macrophages in response to inflammatory cytokines such as TNFα, IL-6, and IFNγ (62, 63). Given the highly inflammatory milieu of female NWB/NZW F1 mice, including elevated levels of serum GM-CSF, IL-6, and IFNα, it is possible that naturally occurring MDSCs in female lupus-prone mice are induced to differentiate into effector cells with an immune-stimulatory phenotype (64, 65). Alternatively, the inflammatory milieu may promote MDSC-dependent extracellular trap formation (and hence MDSC-driven access to nuclear antigens), as suggested by Vlachou et al. (66), although more studies are needed to demonstrate conditions conducive for this change. Interestingly, although studies of MDSCs in SLE patients are few, it has been suggested that MDSCs (as identified by surface markers) from female SLE patients are pathogenic via the production of reactive oxygen species (64, 65). No studies have yet specifically evaluated levels of MDSCs in male SLE patients and matched healthy controls.

TESTOSTERONE AND pDC-DERIVED IFNα AS REGULATORS OF IMMUNOSUPPRESSIVE CELLS?

Similarly to a role for IFNα in inhibiting Tregs and Bregs (see above), IFNα-driven IRF7 was shown to negatively control granulocytic MDSC levels (67), suggesting that IFNα blocks MDSC development (see **Figure 1**). Plasmacytoid dendritic cells (pDCs) are known for their production of IFNα and IL-6 and their role during anti-viral responses. Although not uniform among all SLE patients, elevated levels of type I interferons (IFNα) and the presence of an interferon-stimulated gene signature in PBMCs from SLE patients, further suggest a critical role for pDCs in lupus pathogenesis. Furthermore, IFNα has been implicated a pathogenic role in most animal models of lupus [reviewed in Zhuang et al. (68)] and IL-6 have repeatedly been associated with disease incidence and activity in NZB/NZW F1 mice (69, 70). Interestingly, production of IFNα and IL-6 can be induced by the activation of toll like receptors 7 and 9 on pDCs in response to RNA/DNA containing immune complexes (71), commonly found in SLE serum. IL-6 can also be produced by monocytes, and an *in vitro* study of SLE-derived PBMCs, showed a down-regulation of IL-6 production in response to testosterone treatment (72).

As a product of hematopoiesis, pDCs are likely subject to regulation by sex hormones, although to our knowledge, this has not been specifically evaluated in SLE patients. In healthy adults, however, female-derived pDCs have been shown to produce significantly more IFNα after *in vitro* TLR7 stimulation than male-derived pDCs, although no difference in the number of these cells was found between the sexes (71). Similarly, *in*

vitro studies of pDCs isolated from human infants showed significantly lower IFNα production by male cells than by female cells, a pattern that was even more pronounced if the cells were pretreated with dihydrotestosterone prior to simulation (73).

DISCUSSION

Overall, our understanding of lupus development has improved over the years, but work remains so that better targeted therapies can be developed. The studies discussed in this review highlight that while testosterone itself is unsuitable as a therapeutic agent, downstream targets of androgens represent *de novo* areas for research. Given the clear suppressive effects of androgens on the immune system, of particular interest is pinpointing direct steps in lupus pathogenesis at which testosterone acts, as these may ultimately become areas at which to target new therapies that would maximize disease control while minimizing unwanted side effects. Of particular interest is understanding the mechanism(s) through which testosterone affects numbers and functionality of immunoregulatory cells such as MDSCs, Tregs, Bregs, and M2 macrophages to maintain immunosuppression in genetically predisposed male mice. For example, the direct effect of testosterone on pDCs and IFNα production in combination with testosterone-driven myelopoiesis may directly affect MDSC and M2 skewing, while direct binding to the Foxp3 promoter may regulate Tregs and downregulation of BAFF may affect Breg levels and functions in SLE patients and lupus models exhibiting elevated IFNα levels (see **Figure 1**). While therapies targeting pDCs and IFNα, along with autologous Treg treatment, are currently being investigated in SLE patients, MDSCs and Breg are being actively studied as a therapeutic target in a number of cancer treatments but not in SLE. We propose that these cells, in their native immunosuppressive state, should also be evaluated for their therapeutic potential in lupus and other autoimmune disorders.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Androgen Receptors in Epithelial Cells Regulate Thymopoiesis and Recent Thymic Emigrants in Male Mice

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Androgens have profound effects on T cell homeostasis, including regulation of thymic T lymphopoiesis (thymopoiesis) and production of recent thymic emigrants (RTEs), i.e., immature T cells that derive from the thymus and continue their maturation to mature naïve T cells in secondary lymphoid organs. Here we investigated the androgen target cell for effects on thymopoiesis and RTEs in spleen and lymph nodes. Male mice with a general androgen receptor knockout (G-ARKO), T cell-specific (T-ARKO), or epithelial cell-specific (E-ARKO) knockout were examined. G-ARKO mice showed increased thymus weight and increased numbers of thymic T cell progenitors. These effects were not T cell-intrinsic, since T-ARKO mice displayed unaltered thymus weight and thymopoiesis. In line with a role for thymic epithelial cells (TECs), E-ARKO mice showed increased thymus weight and numbers of thymic T cell progenitors. Further, E-ARKO mice had more $CD4^+$ and $CD8^+$ T cells in spleen and an increased frequency of RTEs among T cells in spleen and lymph nodes. Depletion of the androgen receptor in epithelial cells was also associated with a small shift in the relative number of cortical (reduced) and medullary (increased) TECs and increased CCL25 staining in the thymic medulla, similar to previous observations in castrated mice. In conclusion, we demonstrate that the thymic epithelium is a target compartment for androgen-mediated regulation of thymopoiesis and consequently the generation of RTEs.

Keywords: androgens, T cells, thymus, thymic epithelial cells, mice

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INTRODUCTION

Androgens, such as testosterone, are important modulators of the immune system and immune-related disorders (1). Androgens also suppress the number of peripheral T cells in both mice and men (2–5). Testosterone replacement lowers circulating T cells in hypogonadal men to levels equivalent to those of healthy controls (5) and in patients with Klinefelter syndrome, elevated T cell levels were normalized after testosterone supplementation (4).

T cell progenitors are produced in the bone marrow and then enrolled in thymopoiesis, i.e., further proliferation, selection, and maturation of T cells in the thymus. It is well recognized that androgens have a crucial impact on thymus size and contribute to the involution of the thymus taking place during puberty in both mice and humans. In androgen deficient states, both thymus size and thymopoiesis are prominently increased and the thymus involutes upon treatment with androgens (2, 3, 6–12). Recent thymic emigrants (RTEs), i.e., immature T cells that derive from the thymus and continue their maturation to mature naïve T cells in secondary lymphoid organs, are also regulated by androgens; the fraction of RTEs increases in the periphery after castration and/or androgen deprivation therapy of both mice and humans (3, 5). Besides being progenitors to mature T cells, RTEs have distinct properties and may play specific roles in immune disorders (13).

Despite the important actions of androgens on T cell homeostasis, understanding about relevant target cells remains incomplete. In a series of bone marrow transplantation experiments using the Tfm/Y mouse model, chimeric mice lacking a functional androgen receptor (AR) in non-hematopoietic cells showed increased thymus size, while mice lacking a functional AR in bone marrow-derived cells did not (6). It is therefore likely that AR in stromal cells and not in the hematopoietic compartment is important for the AR-mediated effects of androgens on the thymus. This notion has been strengthened by findings that a cell-specific knockout of AR in epithelial cells regulates thymopoiesis (14). Further supporting thymic epithelial cells (TECs) as an important androgen target, recent data suggest that castration of male mice alters the relative numbers of cortical TECs (cTECs) and medullary TECs (mTECs) (15). Given the central role of TECs for many thymic processes (16), we hypothesized that TECs are target cells for androgen-mediated regulation of both thymopoiesis and peripheral RTEs.

In this study, we have utilized the AR knockout (ARKO) mouse model to investigate how the AR mediates the effects of androgens on thymopoiesis and the peripheral T cell pool, using male mice with general- (G-ARKO) as well as T cell-specific (T-ARKO), and epithelial cell-specific (E-ARKO) knockout of the AR. Specifically, we asked the question whether AR in epithelial cells regulate RTEs in secondary lymphoid organs.

RESULTS

Increased Thymopoiesis in G-ARKO Mice

We first studied the effect of general AR depletion on thymopoiesis; mice with a general knockout of the AR (G-ARKO) had increased thymus weight and cellularity compared to littermate (Pgk-Cre$^+$) controls (**Figures 1A,B**). The number of thymocytes was increased at all stages of T lymphopoiesis, including the early double negative (CD4$^-$CD8$^-$) stages (**Figures 1C–H**) as well as more mature double positive (CD4$^+$CD8$^+$) and single positive (CD4$^+$ or CD8$^+$) cells (**Figures 1I–K**).

As G-ARKO mice are both AR- and testosterone-deficient (17), we next castrated G-ARKO and control littermate mice and replaced with a physiological dose of testosterone (17), to distinguish the effects of testosterone deficiency from AR deficiency on thymus weight. While testosterone replacement reduced thymus weight in castrated control mice, it did not affect the thymus weight of G-ARKO mice (**Supplemental Figure 1**), showing that the effect of testosterone on thymus weight is completely AR-dependent.

Unchanged Thymopoiesis in T-ARKO Mice

We next searched for the target cell for the effects of androgens on thymopoiesis. To assess if androgens/AR affect T cell homeostasis through a T cell-intrinsic mechanism, we generated T cell-specific ARKO (T-ARKO) mice using the pLCK-Cre$^+$ construct, and quantified thymocytes in these mice and littermate (pLCK-Cre$^+$) controls. Despite a highly efficient knockout of AR exon 2 *g*DNA in CD3$^+$ T cells (**Supplemental Figure 2A**), T-ARKO mice had unchanged thymus weight and cellularity, and the number of T cell precursors were unaffected by AR-deficiency in T cells (**Supplemental Figures 2B–G**), showing that the enhanced thymopoiesis in AR deficiency is not T cell-intrinsic.

Increased Thymopoiesis in E-ARKO Mice

As factors secreted by the thymic stroma are known to influence the thymic microenvironment to support T lymphopoiesis (18) and the AR is expressed in thymic epithelial cells (TECs) (6), we hypothesized that TECs are targets for AR-dependent actions on T cell homeostasis. Therefore, we generated epithelial cell-specific ARKO (E-ARKO) mice using a K5-Cre$^+$ construct (19), where Cre is expressed under the control of the K5-promotor in epithelial cells [model described in (20)]. Confirming our hypothesis, E-ARKO mice displayed increased thymus weight and cellularity (**Figures 2A,B**) compared to littermate (K5-Cre$^+$) controls. In line with the results in G-ARKO mice, the number of thymocytes was increased in E-ARKO at all stages of T lymphopoiesis (**Figures 2C–F**).

Increased Staining of CCL25 in Thymi of E-ARKO Mice

CCL25 has been shown to be central to the effects of testosterone deficiency on thymopoiesis (9). Therefore, we next studied the E-ARKO effect on the expression of CCL25 using thymic sections. Indeed, compared to control mice, E-ARKO mice showed an increased CCL25-positive area in medulla, but not cortex (**Figure 3**). A similar pattern was found in castrated (testosterone-deficient) mice (**Figure 3**).

TEC Shift in E-ARKO Mice

Recent data suggest that castration of male mice results in relatively reduced cTEC and increased mTEC number (15). Quantifying mTEC (CD45$^-$ EpCAM$^+$ UEA1$^+$ Ly51$^-$) and cTEC (CD45$^-$ EpCAM$^+$ UEA1$^-$ Ly51$^+$) populations (**Figure 4A**), we saw a similar pattern in E-ARKO, i.e., a relative reduction

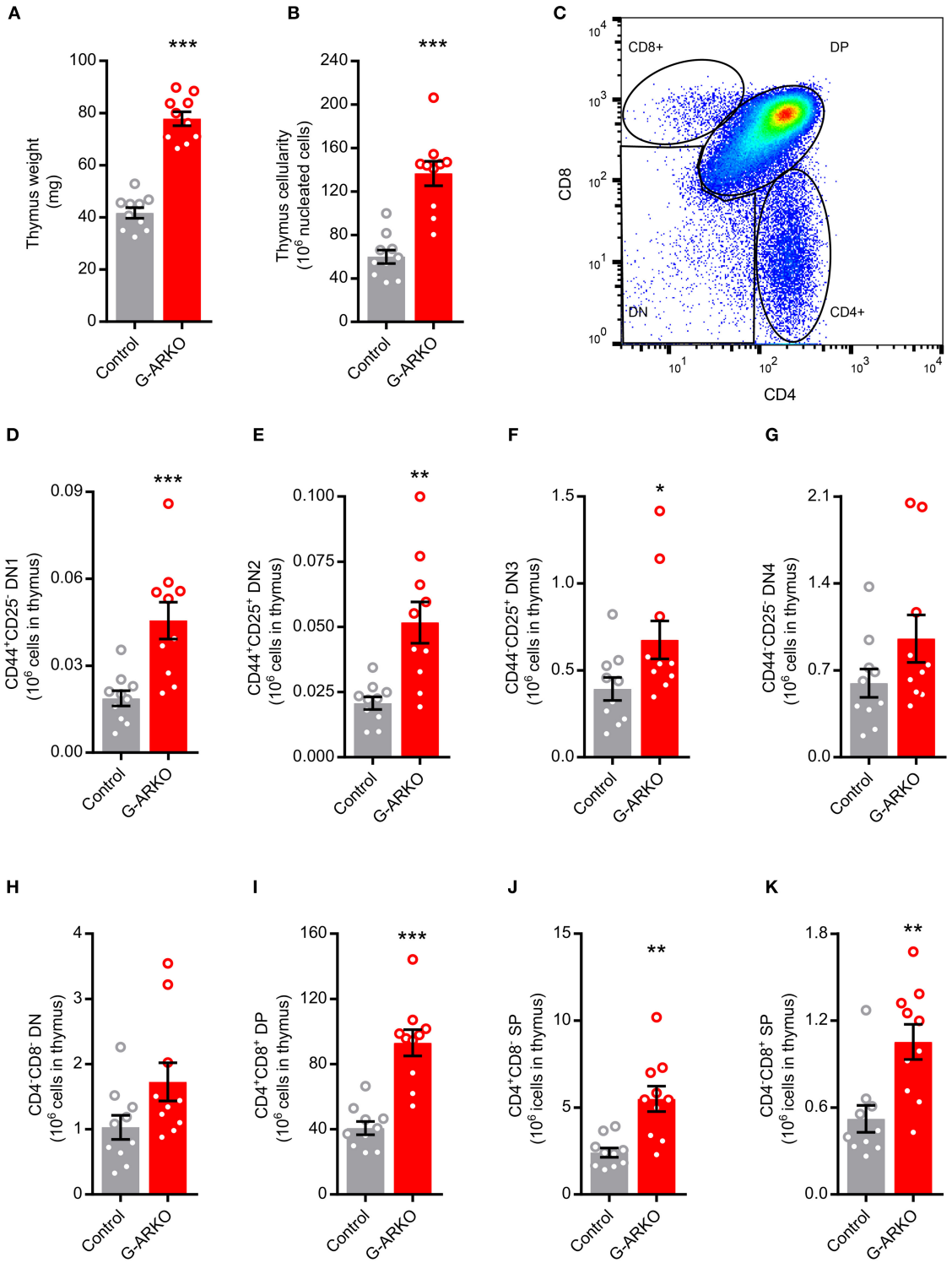

FIGURE 1 | Increased thymopoiesis in mice with general depletion of the AR (G-ARKO). **(A,B)** Thymus weight and cellularity in control (Pgk-Cre$^+$; $n = 10$) and general androgen receptor knockout (G-ARKO; AR^{fl}Pgk-Cre$^+$; $n = 10$) male mice. **(C)** Gating strategy for thymic subsets. **(D–G)** Number of CD4$^-$CD8$^-$ double negative thymocyte subsets (DN1-4, with different expression of CD44 and CD25 markers). **(H–K)** Total double negative (DN), double positive (DP; CD4$^+$CD8$^+$), and single positive (SP; CD4$^+$ or CD8$^+$) thymocytes in control ($n = 10$) and G-ARKO ($n = 10$) mice. *P <0.05, **P <0.01, ***P < 0.001 (Mann-Whitney U-test); all bars indicate means; circles represent individual mice, error bars indicate SEM.

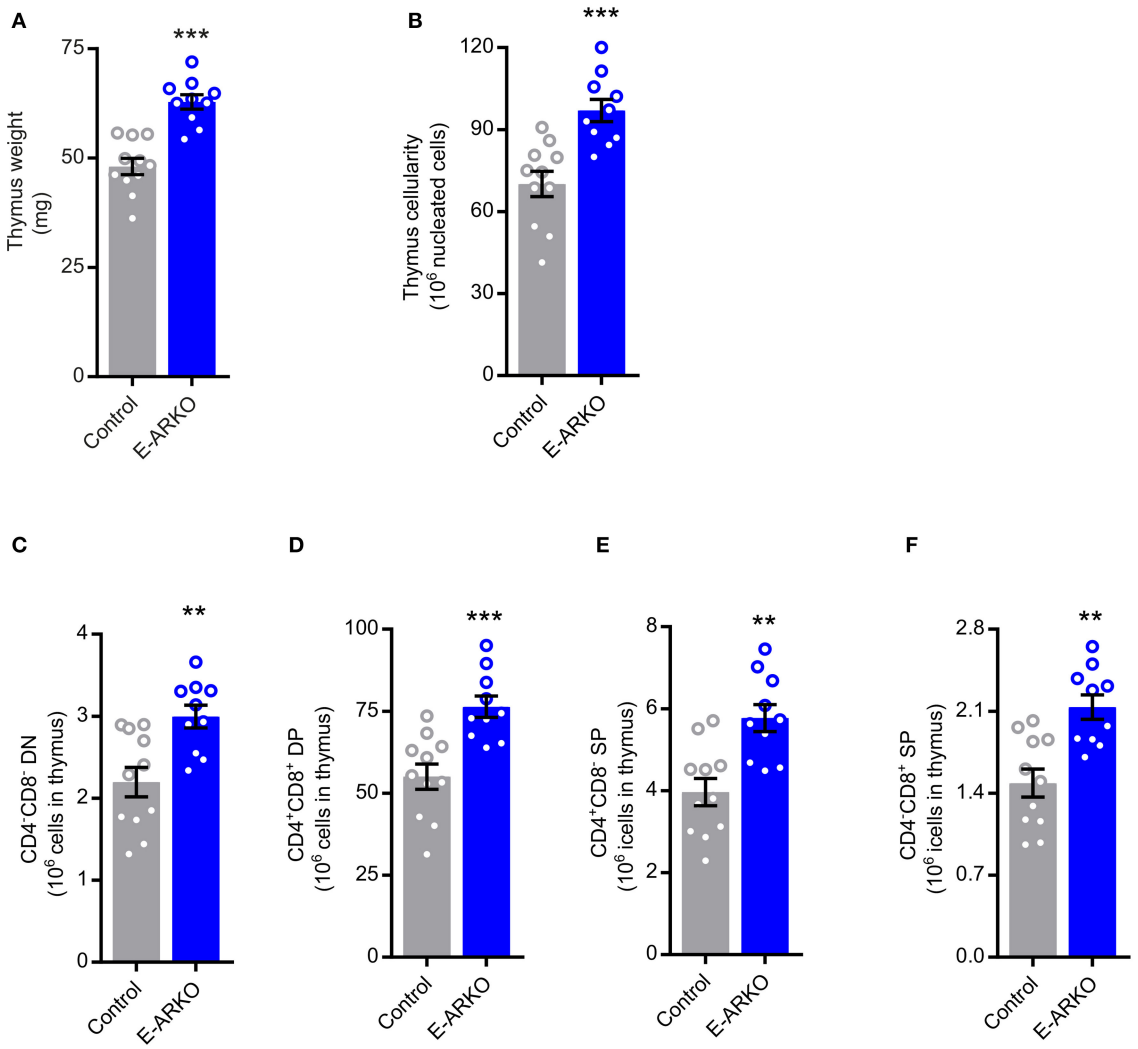

FIGURE 2 | Increased thymopoiesis in mice with depletion of AR in epithelial cells (E-ARKO). **(A,B)** Thymus weight and cellularity in control ($K5\text{-}Cre^+$; $n = 11$) and E-ARKO ($AR^{fl}K5\text{-}Cre^+$; $n = 10$) male mice. **(C–F)** Number of double negative (DN; $CD4^-CD8^-$), double positive (DP; $CD4^+CD8^+$), and single positive (SP; $CD4^+$ or $CD8^+$) thymocytes in control ($n = 11$) and E-ARKO ($n = 10$) mice. $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann-Whitney *U*-test); all bars indicate means; circles represent individual mice, error bars indicate SEM.

of cTECs and a minor increase in the relative number of mTECs. By contrast, the overall TEC fraction in thymus was not different between E-ARKO and controls (**Figures 4B–D**). Analyzing the knockout of AR exon 2 gDNA in mTECs and cTECs, there was a partial (mean 65%) depletion of AR exon 2 in mTECs, but no significant depletion in cTECs (**Figures 4E,F**).

Increased Peripheral T Cells and Recent Thymic Emigrants (RTEs) in E-ARKO Mice

We next studied the peripheral T cell pool and the frequency of RTEs in secondary lymphoid organs of E-ARKO mice. We defined RTEs as $Qa2^{lo}CD24^{hi}$ $CD4^+$ or $Qa2^{lo}CD24^{hi}$ $CD8^+$ cells (13) (**Figure 5A**). E-ARKO showed an increased number of both $CD4^+$ and $CD8^+$ T cells in spleen (**Figures 5B,C**). In E-ARKO mice, RTEs constituted a significantly greater part of total $CD4^+$ (+10%, $P < 0.05$), with a similar trend for $CD8^+$ (+15%, $P = 0.13$) T cells in spleen (**Figures 5D,E**). The total number of both $CD4^+$ and $CD8^+$ RTEs were increased in spleen of E-ARKO mice (**Figures 5F,G**).

In pooled inguinal and para-aortic lymph nodes, the frequency of both $CD4^+$ (+33%, $P < 0.01$) and $CD8^+$ RTEs (+61%, $P < 0.001$) were increased in E-ARKO compared to control mice (**Figures 5H,I**). In blood, the frequency of $CD8^+$ RTEs (+94%, $P < 0.01$), but not $CD4^+$ RTEs (+37%, $P = 0.15$) were significantly increased (**Figures 5J,K**). Taken together, peripheral RTEs were increased in E-ARKO mice, with some variation in effect between compartments.

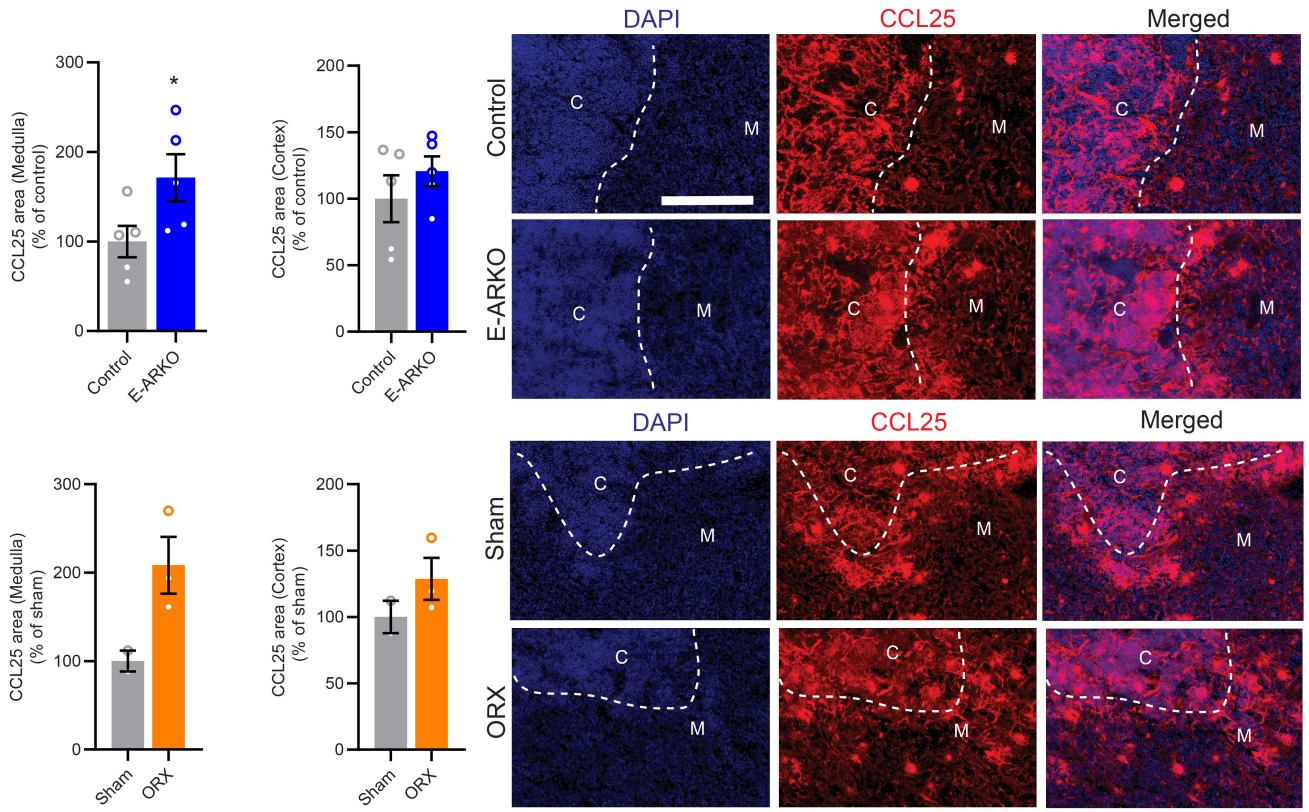

FIGURE 3 | Increased staining of CCL25 in thymi of E-ARKO mice. Quantification of CCL25-stained area in thymic medulla and cortex of E-ARKO and control mice and mice that were sham-operated or castrated (orchiectomized; ORX) at 4 weeks of age; tissues were collected at 34 weeks of age. Thymic sections were stained for CCL25 (red) and nuclei were stained by 4',6-Diamidino-2-Phenylindole (DAPI; blue). Scale bar = 200 μm. *$P < 0.05$ vs. control (Mann-Whitney *U*-test). Bars indicate means, error bars indicate SEM, and circles represent individual mice.

DISCUSSION

In this study, we have utilized cell-specific AR knockout mouse models to investigate how androgens/AR affects T cell homeostasis. We show that epithelial cells are a target for androgen/AR-mediated actions on thymopoiesis, splenic T cells, and RTEs in secondary lymphoid organs.

Here we identified the TEC as a target cell for androgen regulation of thymus size, in accordance with data of increased thymopoiesis in both G- and E-ARKO models (14). Our data are also in accordance with data demonstrating a hematopoietic cell-extrinsic, rather than -intrinsic, AR-dependent inhibition of thymopoiesis (6). In line with the previous data (14), we found that all thymic T lymphocyte stages, from double negative through single positive cells, were increased in both G- and E-ARKO mice.

Notably, the effect size differed between the two models; G-ARKO had a doubling of thymus cellularity, whereas thymus cellularity was increased by ∼40% in E-ARKO. This divergent effect size might result from an incomplete knockout of the AR in TECs of E-ARKO mice as shown here. Further, recent data support that complete androgen blockade affects thymus cellularity partly by increasing the number of thymus-seeding precursors from the bone marrow (21). This bone marrow effect of androgens/AR may also contribute to the discrepancy between the two models, as G-ARKO mice are both AR- and testosterone-deficient due to underdeveloped testes in this model, while E-ARKO mice have normal testicular development and unaltered levels of testosterone (17, 20). In an effort to distinguish the effects of testosterone- vs. AR-deficiency in G-ARKO mice, we castrated the mice to remove endogenous testosterone production and supplemented the mice with a physiological dose of testosterone (17). Our results show that the effect of testosterone on thymus size is completely AR-dependent.

In the present study, we found that the RTEs in secondary lymphoid organs (spleen and lymph nodes) were increased in E-ARKO mice, which has not been reported previously. Overall, these data suggest that TECs are androgen target cells for the regulation of both thymus size and export of RTEs. Indeed, thymus size is an important determinant of thymic output of RTEs, independently of other factors such as age (22). Our data are in line with previous studies suggesting that the fraction of RTEs increases in the periphery after castration and/or androgen deprivation therapy of both mice and humans (3, 5). As RTEs are precursors to mature T cells, the increased frequency of RTEs is a

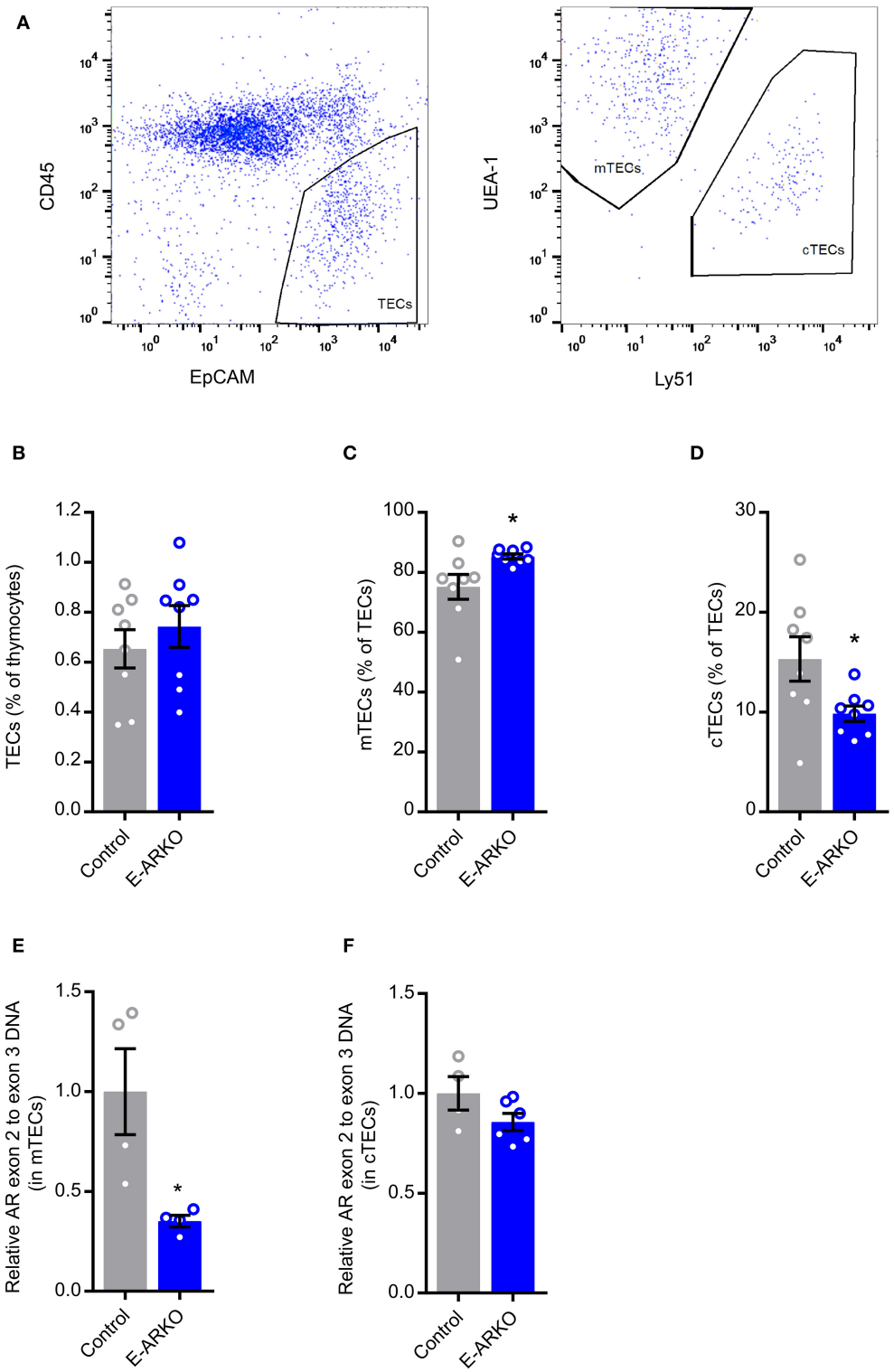

FIGURE 4 | TEC shift in E-ARKO mice. **(A)** Gating strategy for TECs. **(B–D)** Relative numbers of all thymic epithelial cells (TECs), cortical (cTEC; CD45$^-$ EpCAM$^+$ UEA1$^-$ Ly51$^+$), and medullary (mTEC; CD45$^-$ EpCAM$^+$ UEA1$^+$ Ly51$^-$) TEC in control and E-ARKO male mice; $n = 8$/group. **(E)** Assessment of AR knockout by measurement of exon 2 gDNA in mTECs from 4-week-old control (K5-Cre$^+$; $n = 4$) and E-ARKO (AR^{fl}K5-Cre$^+$; $n = 4$) male mice. **(F)** Assessment of AR knockout by measurement of exon 2 gDNA in cTECs from 8-week-old control (K5-Cre$^+$; $n = 4$ pools, 2 mice/pool) and E-ARKO (AR^{fl}K5-Cre$^+$; $n = 6$ pools, 2 mice/pool) male mice. *$P < 0.05$ (Mann-Whitney U test); all bars indicate means; circles represent individual mice, error bars indicate SEM.

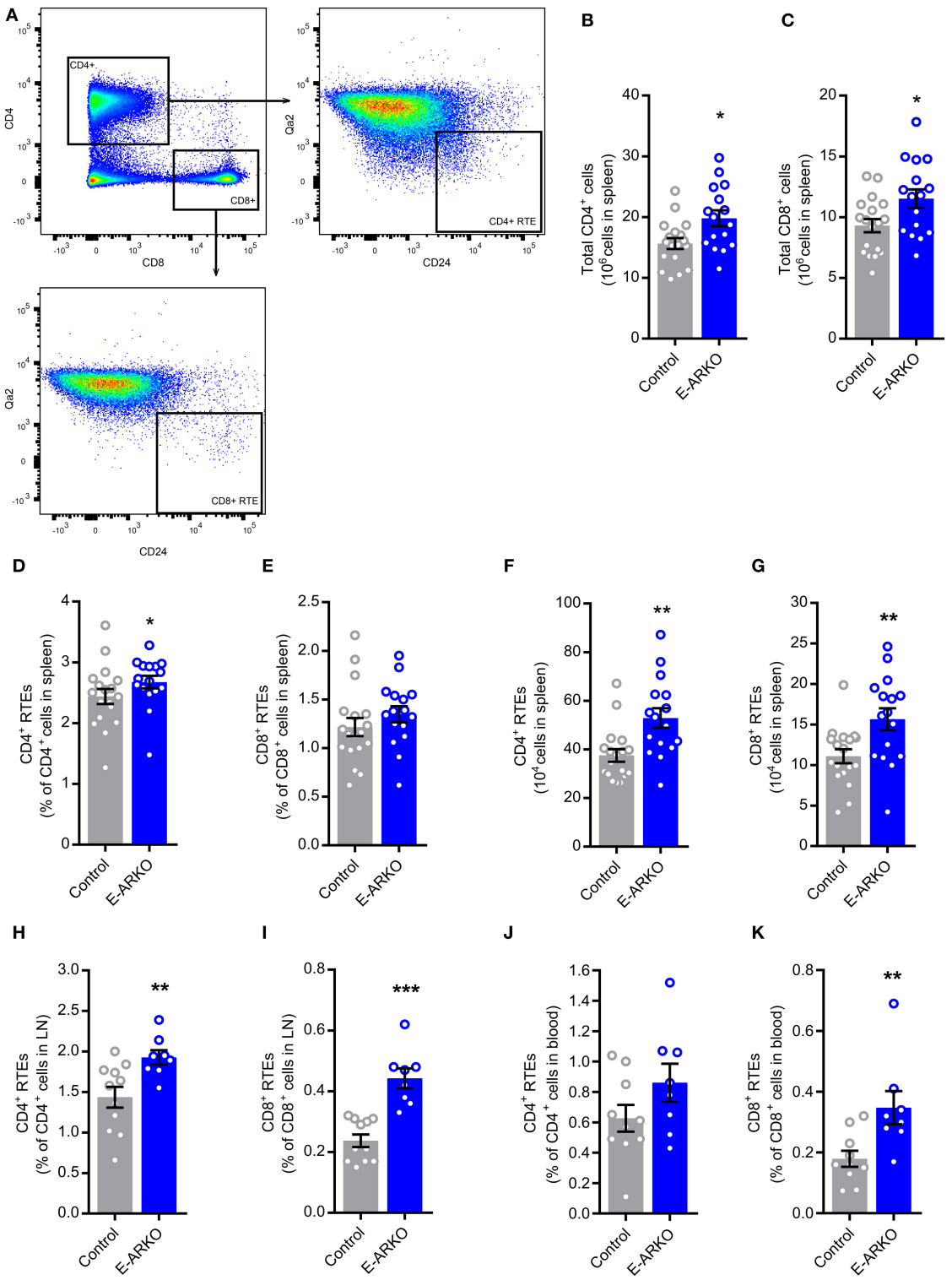

FIGURE 5 | Increased numbers of peripheral T cells and recent thymic emigrants (RTEs) in E-ARKO mice. **(A)** Gating strategy for $CD4^+$ and $CD8^+$ T cells and $CD4^+$ and $CD8^+$ RTEs. **(B,C)** Total numbers of $CD4^+$ and $CD8^+$ T cells in spleen from control ($n = 18$) and E-ARKO ($n = 15$) male mice. **(D–G)** Relative and total number of $CD4^+$ and $CD8^+$ RTEs ($Qa2^{lo}CD24^{hi}$ $CD4^+$ or $Qa2^{lo}CD24^{hi}$ $CD8^+$) in spleen from control ($n = 18$) and E-ARKO ($n = 15$) mice. **(H,I)** Relative number of $CD4^+$ and $CD8^+$ RTEs in lymph nodes (LN; pooled inguinal and para-aortic lymph nodes) from control ($n = 11$) and E-ARKO ($n = 8$) mice. **(J,K)** Relative number of $CD4^+$ and $CD8^+$ RTEs in blood from control ($n = 10$) and E-ARKO ($n = 8$) mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann-Whitney *U*-test); all bars indicate means; circles represent individual mice, error bars indicate SEM.

plausible explanation for the increased number of splenic T cells in E-ARKO mice, which also were found by Lai and coworkers (14). Further, E-ARKO mice showed enhanced donor-derived thymocyte and splenocyte numbers after bone marrow transplantation (14).

Expressed as the percentage out of the total pool of T cells, we found that the frequency of $CD4^+$ RTEs (among $CD4^+$ T cells) was increased in spleen of E-ARKO mice, with a similar trend for $CD8^+$ RTEs (among $CD8^+$ T cells). In the lymph nodes and blood, there seems to be a slightly larger E-ARKO effect on $CD8^+$ RTEs as compared to $CD4^+$ RTEs. Further, there may be a slightly more prominent effect in the lymph nodes as compared to the spleen for both $CD4^+$ and $CD8^+$ RTEs. Although the latter trends require confirmation, they raise the question whether AR depletion in TECs alters the peripheral trafficking of RTEs by shaping RTE properties. To our knowledge, no studies have yet addressed possible androgen/AR-mediated regulation of the homing patterns of RTEs or regulation of their expression of adhesion molecules, chemokines, and/or chemokine receptors known to be involved in the migration of RTEs to secondary lymphoid organs (13).

We used K5-driven Cre recombinase expression to target the AR in TECs. Few studies have reported the degree of DNA depletion in TECs using the K5-Cre construct, as the Foxn1-Cre system has been most widely used for TEC-specific targeting (16). However, using K5-Cre, Lai et al. detected Cre reporter signal in both cortex and medulla (14). In the present study, we found a mean knockout degree of around 65 % in mTECs of E-ARKO mice, while we could not demonstrate a deletion of AR in cTECs. In addition to most differentiated mTECs, common thymic epithelial stem/progenitor cells express K5 (23), but it is possible that K5 expression in early TECs is not general and/or sufficient to drive Cre expression that results in depletion across TEC subsets. Notably, a recent study reported that castration results in reduced cTEC numbers, which the authors coupled to increased apoptosis of cTECs and increased differentiation of cTEC-phenotype progenitors into mTECs (15). Theoretically, if AR depletion in TECs affects cTEC survival and/or differentiation, quantification of AR knockout degree in cTECs may be biased by preferential loss of AR-depleted cTECs/cTEC-phenotype progenitors through these processes. As previously reported after castration (15), we found a shift in the relative number of mTECs and cTECs in E-ARKO mice; thus, the effect of androgen deficiency on mTEC/cTEC ratio is partially mimicked by AR depletion in TECs using K5-driven Cre expression. However, our result contrasts that of Lai et al. who found proportionally increased cellularity of mTEC and cTEC fractions in E-ARKO mice, although they used slightly different markers for defining cTECs (14).

Our finding of no detectable AR deletion in cTECs of E-ARKO mice is unexpected given the thymopoiesis phenotype of these mice, as cTEC functions are known to be important for T cell expansion and development (16). However, molecular delineation of individual TEC subpopulations is a work in progress (16), and new data emphasizes the role of medullary stromal signals for the function of the cortical stroma (24), suggesting an important interplay between different TEC fractions. Notably, we found increased staining of CCL25 in the medulla, but not cortex, of E-ARKO mice, similar to the pattern of castrated (testosterone-deficient) mice. CCL25 has been shown to be an important mediator of the effects of testosterone deficiency on thymopoiesis through increased uptake of early T-lineage progenitors and regulation of the maturation of double negative thymocytes (9). Thus, these mechanisms may possibly be mimicked in the E-ARKO model. Our findings indicate that the androgen/AR-mediated regulation of CCL25 mainly occurs in the medulla, which is in accordance with previous data showing that the greatest increase in CCL25 production after castration is by UEA^+ mTECs (9).

Patients with androgen deficiency, such as Klinfelter patients, have both increased number of T cells (4) and increased risk of autoimmune diseases (25). However, whether androgen-mediated modulation of thymus/TEC biology plays a role in T cell-dependent disorders remains largely unclear. Our group recently showed that E-ARKO mice display increased atherosclerosis, which was abolished by prepubertal thymectomy (20). Although the mechanism underlying increased thymus-dependent atherosclerosis in E-ARKO mice remains to be established, it may theoretically relate to various TEC functions such as negative selection, regulatory T cell formation, or RTE formation (26). To date, it remains unclear whether the E-ARKO mice are prone to other inflammatory and/or autoimmune disorders. Deciphering whether the thymic epithelium is a target compartment for androgen/AR-mediated regulation of inflammation and autoimmunity, and defining the mechanisms mediating such effects, will be important tasks for future studies.

In conclusion, we demonstrate that the thymic epithelium is a target compartment for androgen/AR-mediated regulation of thymopoiesis and consequently the generation of RTEs.

METHODS

Animals

G-ARKO and E-ARKO male mice were generated as previously described (17, 20). T-ARKO and E-ARKO male mice were generated by breeding $AR^{+/flox}$ female mice with male pLCK-Cre^+ mice (Stock no. 003802, B6.Cg-Tg(LCK-Cre)548Jxm/J, Jackson laboratory, Bar Harbor, Maine, USA), and K5-Cre^+ mice (19), respectively. Because our initial assessments of androgen status (wet weight of androgen sensitive organs) and thymus weight and cellularity revealed no differences between AR^+ and AR^{flox} males, Cre^+ littermates without the AR^{flox} construct were used as controls for subsequent experiments. In all experiments the different ARKO mice were compared to littermate controls and the mice were on a C57BL/6 ApoE constitutive knockout background (B6.129P2-Apoetm1UncN11, Taconic). We assessed AR, Cre, and Zfy (for gender) by PCR amplification of genomic DNA (gDNA) (27). The mice were housed in a temperature- and humidity-controlled room with a 06:00–18:00 h light cycle and consumed a soy-free diet (R70, Lantmännen) and tap water *ad libitum*. All animal studies were conducted in compliance with local guidelines and The Ethics

Committee on Animal Care and Use in Gothenburg approved all procedures.

Castration and Testosterone Replacement

In a separate experiment, G-ARKO mice and littermate controls were bilaterally orchiectomized and implanted subcutaneously with a small slow-releasing pellet containing placebo or a physiological dose of testosterone (25 μg/day; Innovative Research of America, Sarasota, FL, USA) as previously described (17).

Tissue Collection

At 10–16 weeks of age (unless otherwise stated), the mice were anesthetized and blood was drawn from the left ventricle and collected in EDTA tubes (Microvette, Sarstedts). The mice were perfused with saline under physiological pressure and tissues (thymus, spleen, inguinal lymph nodes, and para-aortic lymph nodes) were dissected and kept in PBS on ice.

Cell Preparation and Flow Cytometry Analysis of T Lymphocytes

Single cells from thymus, spleen, and lymph nodes were prepared by passing the tissue through a 40 μm cell strainer (Thermo Fisher) using PBS and a syringe plunger. Erythrocytes in blood and spleen were lyzed in lysis buffer (0.16 M NH_4Cl, 0.13 M EDTA, and 12 mM $NaHCO_3$), the cells were washed in flow cytometry buffer (2% fetal bovine serum and 2 mM EDTA in PBS) and counted in an automated cell counter (Sysmex). After FcR-blockage (anti-mouse CD16/CD32, BD Biosciences), antibodies specific for the following markers were used: CD4 (GK1.5, Biolegend or RM4-5, BD Biosciences), CD8a (53-6.7, Biolegend), CD44 (IM7, Biolegend), CD25 (PC61, BD Biosciences), CD24 (M1/69, BD Biosciences), and Qa-2 (1-1-2, BD Biosciences). Immunostained cells were analyzed on a FACS Canto II, Accuri C6, or FACS Aria (BD Biosciences). Data were analyzed using FlowJo (Tree Star) and fluorochrome-minus-one staining was used as controls.

Thymus Sections

Cryosections (10 μm) of thymus were air dried for 2 hrs and stored at −20°C. Sections were fixed for 5 min in 2% formaldehyde, permeabilized with 0.1% Triton-X for 4 min, blocked in 1% bovine serum albumin and Fc-receptor blocking antibody (anti-mouse CD16/32, clone 2.4G2; BD Biosciences; 1:100) for 30 min at r.t. and incubated with a primary polyclonal goat anti CCL25 antibody (InVitrogen; cat no. PA5-47662; 1:500) in blocking buffer overnight at 4°C. Sections were then washed 3 × 5 min in PBS, incubated with secondary antibody F(ab)2 AF594-conjugated donkey anti-goat IgG (Jackson ImmunoResearch; 1:300) in blocking buffer for 1.5 hrs at r.t, stained in 1 μg/mL DAPI in PBS for 3 min, washed as above and mounted with ProlongGold mounting medium (Life Technologies). The thymic medulla and cortex areas were manually delineated by a blinded observer and the CCL25 positive area was quantified using Visiopharm Integrator System (version 2017.2).

Cell Preparation and Flow Cytometry Sorting of Thymic Epithelial Cells (TECs)

The thymi were fragmented and excess of thymocytes washed away by mechanical disruption. TECs were released by enzymatic digestion. Briefly, the thymic fragments were incubated in digestion medium (0.5 U/mL Liberase TM (Roche), 0.2 mg/mL DNase I (Roche) in DMEM/F12) at 37°C with gentle mixing for 20 min. The released cells were transferred into cold flow cytometry buffer. New pre-warmed digestion medium was added to remaining thymic fragments for two more consecutive incubations, to completely dissolve the tissue. The released cell fractions were filtered through a 100 μm cell strainer (BD Biosciences), washed and counted. Cells from the two latter fractions were pooled for analysis. After incubation with FcR block (CD16/CD32, BD Biosciences), antibodies against CD45 (30-F11, BD Biosciences) and EpCAM/CD326 (G8.8, BD Biosciences), Ly51 (6C3, BD Biosciences) and the biotinylated lectin UEA-1 (Vector Laboratories) were added. The cells were washed, resuspended in flow cytometry buffer, and filtered through a 100 μm cell strainer. mTECs ($CD45^-$ $EpCAM^+$ $UEA1^+$ $Ly51^-$) and cTECs ($CD45^-$ $EpCAM^+$ $UEA1^-$ $Ly51^+$) were sorted on a SY3200 cell sorter (SONY Biotechnology Inc.).

AR DNA Quantification

In the ARKO mouse model exon 2 of the AR gene is excised (27) and the presence of exon 2 versus exon 3 was used to quantify the efficacy of the AR knockout. $CD3^+$ cells were isolated from thymus using positive selection with MACS (magnetic-activated cell sorting) CD3 microbeads (Miltenyi Biotec). Genomic DNA from $CD3^+$ cells was isolated using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Genomic DNA amplification was detected using SyBR green master mix (Applied Biosystems) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The following primer pairs were used: AR exon 2, forward GGACCATGTTTTACCCATCG and reverse CCACAAGTGAGAGCTCCGTA; and AR exon 3, forward TCTATGTGCCAGCAGAAACG and reverse CCCAGAGTCATCCCTGCTT. Ct values for AR exon 2 were normalized to Ct values for AR exon 3 using the $2^{-\Delta\Delta ct}$ method (28).

Statistics

Statistical evaluations were performed with Prism software (GraphPad Software, Inc.). The non-parametrical Mann-Whitney *U*-test was used for all for two-group comparisons and Kruskal-Wallis followed by Mann-Whitney U for comparisons between four groups. *P*-values of < 0.05 were considered statistically significant. Unless otherwise specified, results are represented as mean ± SEM.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee on Animal Care and Use in Gothenburg.

AUTHOR CONTRIBUTIONS

AW, ML, IJ, ES, AS, SL, JF, PF, HC, OE, and ÅT designed the studies. AW, ML, IJ, ES, AS, and JF conducted experiments and/or acquired data. AW, ML, IJ, AS, SL, and ÅT analyzed the data. AW, ML, IJ, and ÅT wrote the manuscript. AW, ML, IJ, ES, AS, SL, JF, PF, HC, OE, and ÅT revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01342/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Impact of Androgens on Inflammation-Related Lipid Mediator Biosynthesis in Innate Immune Cells

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Rheumatoid arthritis, asthma, allergic rhinitis and many other disorders related to an aberrant immune response have a higher incidence and severity in women than in men. Emerging evidences from scientific studies indicate that the activity of the immune system is superior in females and that androgens may act as "immunosuppressive" molecules with inhibitory effects on inflammatory reactions. Among the multiple factors that contribute to the inflammatory response, lipid mediators (LM), produced from polyunsaturated fatty acids, represent a class of bioactive small molecules with pivotal roles in the onset, maintenance and resolution of inflammation. LM encompass pro-inflammatory eicosanoids and specialized pro-resolving mediators (SPM) that coexist in a tightly regulated balance necessary for the return to homeostasis. Innate immune cells including neutrophils, monocytes and macrophages possess high capacities to generate distinct LM. In the last decades it became more and more evident that sex represents an important variable in the regulation of inflammation where sex hormones play crucial roles. Recent findings showed that the biosynthesis of inflammation-related LM is sex-biased and that androgens impact LM formation with consequences not only for pathophysiology but also for pharmacotherapy. Here, we review the modulation of the inflammatory response by sex and androgens with a specific focus on LM pathways. In particular, we highlight the impact of androgens on the biosynthetic pathway of inflammation-related eicosanoids in innate immune cells.

Keywords: neutrophils, lipid mediators, 5-lipoxygenase, testosterone, leukotriene, sex

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INTRODUCTION

Inflammation is a protective response mounted by the host immune system against invading pathogens, foreign bodies, or injuries. It is a highly coordinated, active process in order to remove the harmful stimulus and to repair damaged tissues for reestablishing homeostasis. But if an acute inflammatory response fails to resolve, it may contribute to organ pathologies and promote many widespread chronic inflammatory clinical phenotypes such as arthritis, neurodegenerative diseases, asthma, allergy, diabetes, organ fibrosis, cancers, and various cardiovascular disorders (1). Among the plethora of mediators that regulate inflammation in a spatial and temporal fashion, lipid mediators (LM) are crucial molecules that can act at the four major stages of the inflammatory process: initiation, maintenance, resolution, and the return to homeostasis (2, 3). LM encompass a huge class of highly bioactive oxygenated polyunsaturated fatty acids (PUFAs) that play broad and major roles in health and diseases. Here, we refer to LM that are biosynthesized on demand

mainly from the ω-6 PUFA arachidonic acid (AA), and the ω-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) via initial conversion by cyclooxygenases (COX), lipoxygenases (LOX) and monooxygenases (i.e., CYP450 enzymes) (4, 5). These enzymes may partially act in conjunction, and can be coupled to epoxide hydrolases, glutathione transferase and other enzymes to produce defined LM with specific biological functions by acting at select G protein-coupled receptors (GPCR) or nuclear receptors (6, 7). Whereas, most of the AA-derived prostaglandins (PG) and leukotrienes (LT) formed by the COX and 5-lipoxygenase (5-LOX) pathway, respectively, exhibit rather pro-inflammatory properties (3, 8), the so-called specialized pro-resolving mediators (SPM) including lipoxins (LX), protectins (PD), resolvins (Rv), and maresins (MaR) are generated from AA, EPA and DHA with CYPs and 12/15-LOX as key enzymes and promote resolution of inflammation (2, 9).

The responses of the innate and adaptive immune system to foreign and self-antigens differ between females and males, where both genes (on X- and Y-chromosomes) and sex hormones are involved (10, 11). Thus, the age is an important variable where certain immune responses dominate in boys vs. girls before puberty with an opposite bias in adults where the immune system is more pronounced in females (11, 12). Most sex-based immunological differences are obvious after puberty and before reproductive senescence, indicating a crucial impact of sex hormones. These sex differences contribute to divergences in the incidence of autoimmune diseases and other inflammation-related malignancies with implications for both disease pattern and therapy (11). The sex steroids are known to have direct effects on the immune response. They comprise estrogens, progesterone and androgens and are mainly biosynthesized by the gonads of adult females and males. Estrogens (i.e., estrone, estriol, and the most bioactive estradiol) are produced by ovarian granulosa cells in females by the action of the aromatase complex from androstenedione or testosterone. Effects of estrogens on the immune system have often been connected to a pro-inflammatory phenotype (13) although their "stimulatory" action on macrophages results into promotion of the resolution of inflammation (14). Progesterone, besides its well-known essential activity in pregnancy, has been reported to have anti-inflammatory properties by influencing the immune system (15). More recent findings showed that progesterone suppresses LT biosynthesis in human monocytes by inhibition of 5-LOX activity (16). Androgens exert several inhibitory effects on immune cell activity with predominantly anti-inflammatory properties (17). Thus, the androgens testosterone and 5α-dihydrotestosterone (5α-DHT) are highly potent sex hormones that affect a variety of innate and adaptive immune responses with suppressive effects on macrophages, neutrophils, natural killer (NK) cells and T cells (12, 17, 18). For example, testosterone reduces inflammatory reactions such as toll-like receptor (TLR)4 expression on neutrophils, the biosynthesis of TNFα and iNOS-derived NO in macrophages, and NK cell activity (i.e., interferon (INF)γ secretion). On the other hand, androgens can promote anti-inflammatory processes such as expression of peroxisome proliferator-activated receptor (PPAR)α in T cells as well as IL-10 and transforming growth factor (TGF)β formation (11, 18).

Sex differences in PG formation were reported in 1972, where male human subjects were found to excrete larger amounts of the major urinary metabolite 7α-hydroxy-5, 11-diketotetranor-prostane-1, 16-dioic acid of $PGE_{1/2}$ than female subjects (19). Later, in 1974, androgens were found to modulate PG formation (20, 21). In 2008, a striking suppressive potential of androgens on LT formation in human neutrophils was discovered that is causative for lower LT biosynthesis in male vs. female cells (22). Subsequent intensive research in humans and rodents has accumulated substantial knowledge on the impact of androgens on LM biology, and how this translates into modulation of inflammation, and how it affects anti-inflammatory therapy (23). Here we review the effects of androgens on pro-inflammatory LM biosynthesis and we discuss the role of LM in the interplay between androgen functions in innate immune cells with apparent consequences for the incidence and severity of inflammation.

LIPID MEDIATORS: BIOSYNTHESIS PATHWAYS AND THEIR ROLE IN INFLAMMATION

Innate immune cells including granulocytes, monocytes and macrophages possess high capacities to generate pro-inflammatory but also inflammation-resolving LM. Because of their potent bioactivities and broad impact on multiple functions of the body, LM are not stored in cells or tissues at large amounts but are rather *de novo*-synthesized on demand. The first step in the biosynthesis of LM is the liberation of the fatty acid substrate mainly from membrane phospholipids by phospholipase A_2 (PLA_2) enzymes (8, 24). Thus, modulation of PLA_2 and concomitant fatty acid release as LM substrate may be subject of regulation by androgens and/or sex. However, only little is known on androgens affecting PLA_2 (25, 26) and how this would translate into significant biological effects or sex differences connected to LM.

PGs and thromboxanes (TX)s are generated by COX-1/2 in a two-step conversion of liberated AA which leads to the formation of the instable intermediates PGG_2 and then PGH_2, where the latter is transformed by tissue-specific prostanoid synthases into the different bioactive prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 (**Figure 1**). Two isoforms of COX exist with COX-1 being constitutively expressed in many different cell types, and the inducible COX-2, which is abundantly expressed in pro-inflammatory innate immune cells including monocytes, neutrophils and M1-like macrophages after exposure to inflammatory stimuli (e.g., cytokines, bacterial components or hormones). PGE_2 mediates inflammation-related processes such as vasodilatation and increased microvascular permeability, as well as fever, and pain (27). It is synthesized from PGH_2 by cytosolic PGE synthase (cPGES) or microsomal PGE synthases (mPGES-1 and mPGES-2), with mPGES-1 being an inducible isoform with high relevance for producing PGE_2 during inflammation (28).

LTs have significant impact on inflammatory and immune-related diseases like asthma and allergic rhinitis, autoimmune

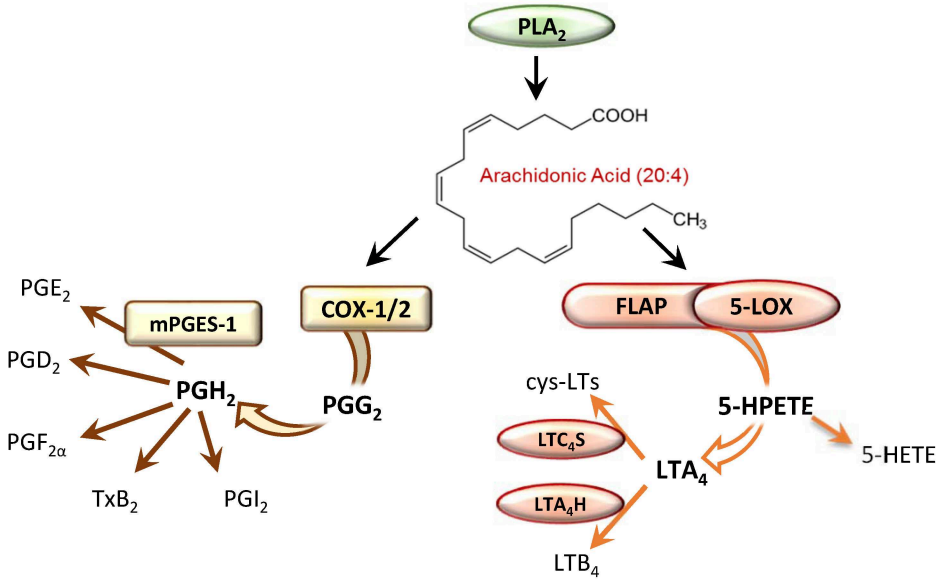

FIGURE 1 | Enzymatic conversion of arachidonic acid by the cyclooxygenase (COX) or the 5-lipoxygenase (5-LOX) pathway leads to formation of prostaglandins (PG) or leukotrienes (LT), respectively.

diseases, atherosclerosis, cardiovascular diseases and cancer (29). The ability to generate LTs is mainly restricted to innate immune cells such as neutrophils, eosinophils, monocytes, macrophages and mast cells that express the required biosynthetic enzymes. For the biosynthesis of LTs, AA is released from phospholipids by the cytosolic PLA_2 and provided to 5-LOX by the nuclear membrane-bound 5-LOX-activating protein (FLAP) (30). 5-LOX then facilitates the incorporation of molecular oxygen at C5 of AA yielding the hydroperoxide 5(S)-HPETE that is converted in a second step by 5-LOX into the epoxide LTA_4 (**Figure 1**). 5-LOX is localized in the nucleoplasm or the cytosol depending on the cell type, and after cell activation it translocates to the nuclear envelope in order to access liberated AA (31). 5-LOX is composed of a catalytic domain, with an iron in the active site, and a regulatory C2-like domain that binds Ca^{2+} and mediates the association with phosphatidylcholine (PC) and thus with PC-rich membranes (30). At the nuclear membrane 5-LOX assembles a complex with FLAP that binds AA and facilitates the accessibility of the substrate for 5-LOX, which is considered pivotal for LTA_4 generation in intact cells (32, 33). Depending on the cell-type and the enzymes expressed, LTA_4 is then converted to LTB_4 by LTA_4 hydrolase (LTA_4H) or to LTC_4 by LTC_4 synthase (LTC_4S) (30) (**Figure 1**). LTB_4 is a potent chemoattractant and activator of various leukocytes whereas cys-LTs mediate smooth muscle contraction and vascular leakage (8).

In contrast to pro-inflammatory PGs and LTs, the SPM have anti-inflammatory properties as they actively terminate inflammation and promote the resolution of the inflammation process. The SPM superfamily encompasses LM with conserved structures mediating their bioactions via distinct GPCR with defined structure-activity relationships (2, 7). SPMs exhibit beneficial functions in microbial defense, pain, organ protection, and tissue regeneration, wound healing, cancer, reproduction, and neurobiology of cognition (9). The generation of LX from AA requires the concomitant actions of 5-LOX and 12/15-LOXs which can be accomplished by single cells that express both LOXs or by transcellular metabolism between cells that express at least one of these enzymes (9, 34). Similarly, the formation of Rv from EPA or DHA depends on at least two enzymes: a CYP450 or COX-2 in the presence of aspirin first generate 18-HEPE from EPA, while in a second step 18-HEPE is converted by a LOX yielding E-series Rv, or in the case of D-series Rv transformation of DHA by 15-LOX to 17-HDHA and its subsequent conversion by 5-LOX and other enzymes (34–36). On the other hand, for the biosynthesis of PD and MaR from DHA, 15-LOX and/or 12-LOX appear sufficient (37). Finally, stereoisomers of LX, Rvs and PD, so-called aspirin-triggered (AT)-LXs, AT-Rvs, and AT-PDs account to the SPM superfamily and are produced by LOXs alone or in conjunction with aspirin-induced acetylated COX-2 (9, 34). To the best of our knowledge, effects of androgens on the biosynthesis of SPM have not been reported yet. Nevertheless, sex differences in SPM levels in humans and mice were recently documented (38–41). Along these lines, the impact of androgens on 12- and 15-LOXs in innate immune cells is essentially unexplored; one study reported that androgen failed to alter 15-LOX-2 gene expression in normal human prostate epithelial cells (42).

SEX DIFFERENCES IN INFLAMMATION AND IN AUTOIMMUNE DISEASES

Numerous studies have shown that sex is a significant variable in the activity of the innate and the adaptive immune system and

that there are significant sex differences in the immune response (11, 43). This affects the defense against infections, autoimmune diseases and tumor diseases as well as vaccinations. Adult women generally have a more active innate and adaptive immune system, explaining why 80% of patients with autoimmune diseases are females, and why women with acute HIV infection have 40% less viral RNA in the blood than men. Moreover, antibody formation in response to influenza vaccines is consistently at least twice as high in women than in men. Age plays a decisive role in this, according to which the immune response and inflammatory activity predominate until puberty in boys, but then dominate in adult women (12). Since sex differences are often caused by divergent levels of androgens, many studies that revealed modulation of the innate immunity by male sex hormones originate from studies primarily aiming to compare biological responses in male and female subjects.

Studies on the mechanisms underlying sex-biased immune responses show quite complex differences in various immune cells between the sexes, both with regard to the innate and the adaptive immunity. For innate immunity, the phagocytosis rate of neutrophils and macrophages, the macrophage activity, the type 1 interferon activity of dendritic cells (DC), and the efficiency of antigen-presenting cells (APC) are all more pronounced in female vs. male cells (44–46). In contrast, the numbers of natural killer cells and the expression of the TLR4 of macrophages and neutrophils dominate in men (11). However, TLR7 is more strongly expressed in women (47). In terms of acquired immunity, women have more B lymphocytes and, accordingly, more pronounced antibody production, higher numbers of activated T lymphocytes with a higher proliferation rate, more CD4+ cells, and more pronounced T cell cytotoxicity. Men, on the other hand, have higher numbers of regulatory T lymphocytes and CD8+ cells (11).

Due to the higher activity of the immune system in women, inflammatory reactions occur more frequently and more severely in females than males. Distinct sex differences can be found in the incidence of autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), thyroid disease, scleroderma or rheumatoid arthritis, but also Alzheimer's disease, which mostly dominate in women (48). In the US, women represent ∼80% of all cases of autoimmune disorders. Also in animal models of autoimmune disease such as experimental autoimmune encephalomyelitis (EAE), females show more pronounced severity (49) with higher activation status of T_H1 cells and elevated INFγ (50). Androgens are protective in the susceptibility to EAE of SJL mice, as castration caused increased severity of the disease and supplementation with exogenous testosterone to both gonadally intact SJL and C57BL/6 mice resulted in protecting effects. Of note, ovariectomy did not alter the disease outcomes (51). Interestingly, administration of sex hormones to patients with MS revealed beneficial effects: in men, topical application of testosterone significantly reduced IL-2 but elevated TGFβ production in PBMC, and shifted the lymphocyte composition by decreasing CD4+ T cells and increasing NK cells (52).

TABLE 1 | Sex differences and modulatory effects of androgens on leukotriene formation in various models and cell/tissue sources.

Species	Model/ Source	Stimulus	Effect of androgens	Male vs. Female	Reference
Human	Whole blood	A23187 fMLP	⇓	M < F	(22, 23)
	Neutrophils	A23187	⇓	M < F	(22, 23)
	Monocytes	A23187	⇓	M < F	(54)
	Emotional tears	–	n.d.	M > F	(38)
Mouse	Peritonitis	zymosan	⇓	M < F	(23, 55)
	Peritoneal macrophages	A23187	n.d.	M < F	(55)
	Lungs	OVA	n.d.	M < F	(56)
Rat	Pleurisy	carrageenan	n.d.	M < F	(23)

SEX BIAS IN PROSTAGLANDIN AND LEUKOTRIENE BIOSYNTHESIS

More than 50 years ago, modulation of eicosanoid biosynthesis by sex was reported (53) and effects of testosterone on $PGF_{2\alpha}$ formation were observed already in 1974 (20, 21). Until today, multiple studies employing different animal models uncovered sex differences and effects of sex hormones on the biosynthetic pathways of PGs and LTs in various cells and organs/tissues (**Tables 1**, **2**), see also (66) for review. LM levels *in vivo* can be affected at the level of their biosynthesis as well as of their metabolism/elimination. Most studies addressed the regulation of the biosynthesis of LM, focusing on the expression of LOXs, COXs or prostanoid synthases as biosynthetic enzymes, or on the cellular activation of these enzymes (66). Notably, also the receptors that induce LM formation (e.g., TLR4) can be strongly modulated by sex and sex hormones (11).

The existence of a sex dimorphism in LT biology is already suggested by the fact that many diseases related to LT including asthma, rheumatoid arthritis, allergic rhinitis, or SLE are sex-biased with higher occurrence in women (66). Similar as for PG formation, modulation of LT production may occur on the expression level of LT-biosynthetic enzymes and via the availability of AA as substrate but also additional regulatory aspects such as modulation of phosphorylation and subcellular redistribution of the biosynthetic enzymes. Note that despite comprehensive and intensive research with the aim to reveal roles of LTs in sex-afflicted autoimmune diseases such as SLE, potential sex differences in LT biosynthesis have long been neglected in biomedical research.

In fact, there is accumulating evidence suggesting that female sex is afflicted with higher LT biosynthesis and androgens were shown to lower LT levels *in vitro* and *in vivo* (**Table 1**) (22, 23, 54–56). For example, blood or isolated monocytes and neutrophils from healthy adult women exhibited higher capacities to produce LTs upon stimulation *ex vivo* vs. men (22, 23, 54). Also, in urine samples from healthy white volunteers, higher concentrations of 5-LOX products were found in samples from elderly women

TABLE 2 | Sex differences and modulatory effects of androgens on prostaglandin formation in various models and cell/tissue sources.

Species	Model/Source	Stimulus	Effect of androgens	Male vs. Female	References
Human	Emotional tears	–	n.d.	M > F	(38)
	Neutrophils	A23187	n.d.	M > F	(57)
	Monocytes	LPS	⇓	n.d.	(58)
	Primary human coronary artery smooth muscle cells (HCASMC)	– LPS IL-1β	⇑ ⇓ ⇓	n.d.	(59)
	HUVECs	LPS TNFα	⇓ ⇓	n.d.	(60)
Mouse	Peritonitis	zymosan	n.d.	M > F	(57)
	Testis, epididymis, vas deferens, and seminal vesicles	–	⇓	n.d.	(61)
Rat	Pleurisy	carrageenan	n.d.	M > F	(57)
	Arthritis	collagen	⇓	M < F	(62)
	Vas deferens, epididymis and the seminal vesicles	–	⇑	n.d.	(20)
	Prostatic and vesicular glands	–	⇓	n.d.	(21)
	Cerebral blood vessels	–	⇑	n.d.	(63, 64)
	Bladder epithelium	–	⇓	n.d.	(65)

than elderly men (67). Such sex-bias was evident also in mice and rats *in vivo*, where female animals in zymosan-induced peritonitis, carrageenan-elicited pleurisy or experimental models of inflammation upon allergen challenge produced higher levels of LTs (23, 55, 56).

Studies with knock-out (KO) animals where 5-LOX or LT receptors were deleted revealed obvious roles of LTs for sex differences in the pathophysiology of diseases. For example, in SLE-related mouse models the female disadvantage vs. male animals was abolished after 5-LOX KO (68). Deletion of the BLT1 receptor protected only female but not male mice in platelet-activating factor (PAF)-induced shock (69) and knockout of the Cys-LT1 reduced tumor burden in the small intestine of female Apc (Min/+) mice but not in male counterparts (70). Conclusively, the superior benefit of female animals after disruption of the LT pathway implies the existence of sex differences in LT biosynthesis with more pronounced pathophysiological roles of LTs in females.

In line with the genetic interference with the 5-LOX pathway, also results from pharmacological studies intervening with LTs imply superior significance of LT biology in women, suggesting that females may benefit from anti-LT therapy to a greater extent than males. In a prospective cohort study with 11 asthmatic women and 10 men, montelukast [a potent and selective leukotriene receptor antagonist (71)] improved asthma symptoms and lung function better in women compared with men (72). Similarly, in asthmatic girls reaching puberty, montelukast clearly reduced asthma incidence vs. placebo, but not so in boys of the same age, even though montelukast was highly active in boys before puberty (73). Along these lines, superior therapeutic efficacy of different types of clinically relevant LT modifiers like zileuton, MK886 and montelukast were observed in female ovalbumin-sensitized BALB/c mice (56). Recent data showed that FLAP inhibitors like MK886 have impaired efficiency in males which is caused by androgens. Thus, lower doses of MK886 were needed to reduce LTB_4 levels in inflammatory exudates of female vs. male mice and rats. Following PAF-induced shock, MK886 increased survival exclusively in female mice, which was abolished by androgen administration. FLAP inhibitors had higher potencies in human blood from females and supplementation of female blood with androgens abolished the observed sex differences (23). Together, the androgen-mediated suppression of LT biosynthesis is not only of pathophysiological relevance for LT-related diseases, but also determines the efficacy of anti-LTs, in particular the potency of FLAP inhibitors which is strikingly impaired by androgens.

Sex differences in PG formation are frequently related to sex-biased expression of the key enzymes involved. Thus, sex-related regulation of COX-2 and mPGES-1 in different organs with consequences for the development and incidence of PG-related diseases was reported (66). In general, prostanoids and their biosynthetic enzymes often dominate in males vs. females, but this can depend on the type of cell, tissue and organ, on the inflammatory status, as well as on the sex hormone level related to the age of the subjects studied. Most studies addressed kidney and brain, where in particular the COX-2/mPGES-1-derived PGE_2 plays major physiological and pathophysiological roles. Three independent studies in rodents addressed sex differences in PG biology of the kidney indicating that female animals reached higher PG (mainly PGE_2) concentrations vs. male counterparts. This bias was either due to superior COX-2 and mPGES-1 levels in females (74, 75) or due to higher expression of the PG-metabolizing enzyme 15-hydroxy-PG dehydrogenase (15-PGDH) and the PG-specific transporter OAT-PG in males (76). In contrast, in the brain, a preponderance of PG

were evident in males (e.g., higher COX-2 expression), and suppressive effects by female sex hormones were reported (77–80). Most strikingly, under inflammatory conditions, for example in mouse zymosan-induced peritonitis and rat carrageenan-induced pleurisy, PG levels dominated in males vs. females, seemingly due to higher PG production in infiltrated male neutrophils that produced more PGE_2 accompanied by elevated COX-2 expression (57). Note that sex differences in prostanoid biology may be caused also by a dimorphism in the expression and function of prostanoid receptors (66). Finally, sex differences in prostanoid biosynthesis and modulation by sex hormones were also observed on the cellular level where cells (e.g., neutrophils, platelets) from male donors generated more prostanoids as compared to female counterparts (38, 57, 81, 82).

IMPACT OF ANDROGENS ON LIPID MEDIATOR FORMATION IN INNATE IMMUNE CELLS

Androgens as Modulators of Innate Immune Cells

Among innate immune cells, neutrophils and monocytes/macrophages are the major source of LM, particularly in the blood. As reported below (section Androgens and Leukotriene Biosynthesis and Androgens and Biosynthesis of Prostaglandins and Other Eicosanoids), the influence of sex hormones and especially of androgens cause significant modifications in the production of LM with consequences for inflammation and possibly also the related pharmacology. There are several findings that indicate a considerable impact of androgens on the count and the functions of these LM-producing innate immune cells with consequences for the immune response. Neutrophils are generated in the bone marrow from myeloid progenitors and play a major role in the first response during inflammation as they represent the early cells recruited to the inflamed tissue to destroy external pathogens (83). It has been shown that the lack of androgen receptor (AR) causes neutropenia and increases the susceptibility to microbial infection (84). Also in a model of ozone-induced airway hyperresponsiveness, androgens caused an increased inflammatory response in terms of neutrophil recruitment as well as cytokine production in an AR-dependent manner where castration of mice diminished the effect (85). Accordingly, in a bacterial model of prostate inflammation, neutrophil accumulation in several organs and in the inflamed region was promoted by testosterone treatment (86). Note that different and contradictory effects of testosterone on neutrophil functions were reported: for example, after trauma-hemorrhagic shock, neutrophils from male mice were more activated then the female counterparts (87), while after bacteria-induced prostate inflammation, neutrophils from male animals produced less pro-inflammatory cytokines (85).

Monocytes and macrophages both express the AR and the effect of testosterone on the functions of those cells is widely discussed (18, 88). Monocytes account for up to ∼10% of the circulating leukocytes in humans and ∼4% in mice and they are evoked during inflammation in large amounts in order to amplify the inflammatory reaction (89). Macrophages that possess high plasticity, can act as pro-inflammatory (M1) or pro-resolving phenotypes (M2), the latter promoting the return to homeostasis (90). Intriguingly, testosterone reduces the expression of TLR4 in murine macrophages (91), but male macrophages have seemingly a superior response to TLR4 ligands (92). Testosterone may also impact macrophage polarization: for example, upon Coxsackievirus B infection, male mice suffered more from myocarditis than females which correlated with a predominance of M2 in female myocardium as compared to increased numbers of M1 in males (93).

Androgens and Leukotriene Biosynthesis

The superior levels of LTs formed in women and female animals or in cells derived from female subjects vs. male counterparts can be unequivocally related to the suppressive impact of androgens. **Table 1** summarizes the reported effects of androgens in various models in relation to the observed sex differences in these studies. The first report demonstrating modulation of LT biosynthesis by androgens *in vitro* was published in 2008 by Pergola et al. (22), almost 30 years after the discovery of LTs by Samuelsson et al. in 1979 (94). Suppression of 5-LOX product formation by androgens like testosterone or 5α-DHT was observed in agonist-stimulated human blood, isolated human neutrophils (22, 23) and monocytes (54) from females. Also in human corneal, conjunctival, and meibomian gland epithelial cells 5α-DHT reduced the potentiation of LPS-induced secretion of LTB_4 via LPS-binding protein (95).

The existence of such androgen effects *in vivo* was confirmed in a murine zymosan-induced peritonitis model (55). Thus, LT levels in peritoneal exudates of orchidectomized mice were higher than in sham male mice, and peritoneal macrophages from orchidectomized animals produced more LTs *ex vivo* than sham-treated counterparts (55). Along these lines, short–term application of 5α-DHT reduced LTB_4 levels during zymosan-induced peritonitis only in female but not in male animals (23). Androgen-mediated sex differences were recently shown also in a mouse model of MS (16), where LT signaling contributes to pathology (96). Moreover, *ex vivo* analysis of blood from healthy women with variant androgen levels revealed a significant invers correlation between androgen levels and the capacity to produce LTs upon stimulation of the blood (22).

In leukocytes, suppression of LT formation by androgens required very short pre-incubation periods of only one to few minutes, excluding the requirement of protein *de novo* synthesis, supported by the fact that androgen treatment did not alter the amounts of any of the LT biosynthetic enzymes on the protein level. Also, in neutrophils and monocytes derived from male and female subjects these LT biosynthetic enzymes were equally expressed (22, 54). Moreover, the magnitude of (concentration-dependent) maximal inhibition of LTs was <50% of the total

5-LOX activity, suggesting that androgens do not directly act on 5-LOX like typical enzyme inhibitors. In fact, androgens were not active in male blood or leukocytes and they suppressed 5-LOX product formation in female cells or blood down to the levels obtained in male counterparts. Interestingly, supplementation of these neutrophils or monocytes with exogenous AA as substrate reverted androgen-induced inhibition of 5-LOX, indicating that androgens may inhibit 5-LOX product formation at the level of substrate supply (20). However, 5α-DHT failed to inhibit AA release in these leukocytes, suggesting another point of attack for androgens; seemingly the accessibility of AA to 5-LOX mediated by FLAP is affected by the sex hormone.

For LT formation, soluble resting 5-LOX in the cytosol or nucleosol must translocate to the nuclear envelope upon cell stimulation for associating with FLAP that facilitates access of 5-LOX to endogenous AA as substrate (32, 33). While in female neutrophils, this pattern of 5-LOX subcellular redistribution was evident along with high LT levels, in male neutrophils a substantial part of 5-LOX was located to the nuclear envelope in resting cells, and only a small portion of 5-LOX redistributed upon stimulation, accompanied by low 5-LOX product biosynthesis (22). Treatment of female cells with 5α-DHT caused the same pattern of 5-LOX redistribution like in male neutrophils and suppressed LT biosynthesis in female cells down to the levels obtained by male neutrophils. Note that also $cPLA_2$ is a cytosolic enzyme that co-translocates with 5-LOX to the nuclear envelope where AA as substrate for 5-LOX is liberated from PC (24). However, neither sex differences nor effects of androgens were observed for $cPLA_2$ translocation in neutrophils (22), suggesting that the sex bias in LT formation is independent from $cPLA_2$-mediated substrate supply. On the other hand, sex differences in *n*-3 PUFA content were reported with associated differences in circulating concentrations of *n*-3 PUFA, that is, higher plasma DHA concentrations in females and negatively associated with circulating concentrations of testosterone (83).

More detailed mechanistic analysis on the subcellular localization of 5-LOX uncovered that androgens impede the agonist-induced, tight assembly of the LT-biosynthetic 5-LOX/FLAP complex at the nuclear membrane of human and murine leukocytes (23). By means of a proximity ligation assay that enables the visualization of *in-situ* 5-LOX/FLAP interaction in intact cells (32) it was found that despite perinuclear localization of 5-LOX in male neutrophils, monocytes or peritoneal macrophages, 5-LOX and FLAP hardly associated as compared to female cells. Addition of 5α-DHT to female cells prevented the agonist-induced 5-LOX/FLAP complex assembly as it was observed in male cells (23). In conclusion, androgens may cause perinuclear localization of 5-LOX but distant from nuclear membrane-bound FLAP which impedes 5-LOX product formation.

It was shown that the variant testosterone levels in males and females cause a differential activation status of extracellular signal-regulated protein kinase (ERK)-1/2 in human neutrophils which confers sex differences in LT formation by regulating 5-LOX subcellular localization (22). Because ERKs are central signaling kinases that regulate multiple neutrophil functions, these actions of androgens may have striking effects on neutrophil biology besides regulating LT formation. 5-LO translocation is governed by ERK-1/2 that phosphorylate 5-LOX (97) and it was shown that ERK-1/2 mediate the male pattern of 5-LOX subcellular distribution caused by androgens. Thus, male (resting) neutrophils exhibited a higher ERK-1/2 activation status vs. cells from females, and pharmacological suppression of ERK-1/2 activity in male cells yielded the female 5-LOX subcellular localization pattern. Of interest, exposure of female neutrophils to male plasma or to 5α-DHT enhanced ERK-1/2 activation, accompanied by the male pattern of 5-LOX subcellular distribution, which again was reversed by ERK-1/2 inhibition (22). ERK activation in neutrophils by 5α-DHT was rather rapid, occurring with 0.5 min, at fairly low concentrations, starting at ∼10 pM. Rapid activation of ERK-1/2 by androgens through a non-genomic mechanism was shown before for non-immune PMC42 breast cancer cells (98). Notably, the androgen effects on ERK-1/2 and 5-LOX were reversible and disappeared after about 1 h at room temperature but were preserved for longer times when neutrophils were kept at 4°C (22). This finding implies that isolation of neutrophil and probably also of other blood cells at low temperature, i.e., 4°C, may better maintain the biology of the cells that they once possessed in their original environment.

As mentioned above, in addition to neutrophils, higher 5-LOX product formation (about 1.8-fold) was also found for isolated human monocytes derived from peripheral blood of females vs. monocytes from males (54). Again, resuspension of female monocytes in plasma from males lowered 5-LOX product formation, and preincubation with 5α-DHT for 30 min repressed LT synthesis in female cells down to the levels observed in males, while estradiol and progesterone were inactive or gave only slight inhibition. 5α-DHT caused rapid ERK-1/2 phosphorylation connected to inhibition of phospholipase D (PLD) and reduced diacylglycerol (DAG) formation. It was shown that DAG are of importance for 5-LOX product formation (99) by acting at a phospholipid binding site of 5-LOX located within the C2-like domain (100). In fact, in male monocytes the ERK-1/2 activation status was increased while PLD activity and DAG formation was 1.4–1.8-fold lower vs. female monocytes. Supplementation of monocytes with DAG elevated 5-LOX product formation in male but not in female cells. These results indicate that androgen-induced ERK-1/2 activation represses PLD activity in monocytes resulting in impaired 5-LOX product formation seemingly due to lack of activating DAGs (54).

In addition to androgens, also progesterone was found to down-regulate LT formation in human monocytes in a rapid manner, however, not in neutrophils (101). Progesterone was more effective in monocytes derived from females and suppression of 5-LOX product synthesis was proposed to be mediated by protein kinase A, whereas ERK-1/2 and PLD were seemingly not involved (101), indicating that the characteristics and mechanisms behind the LT-suppressive effects of progesterone and androgens clearly differ. Moreover, estradiol was shown to induce LTC_4 biosynthesis in RBL-2H3 cells and potentiated LTC_4 formation in response to IgE seemingly by promoting intracellular Ca^{2+} mobilization (102). Finally,

pregnancy, a situation with marked alterations in sex hormone plasma levels, was shown to be associated with increased LT biosynthesis in blood due to higher numbers of leukocyte and due to stimulatory effects of plasma components (103). It is interesting in this respect that the characteristics of diseases related to LT (e.g., asthma, allergic rhinitis) change during pregnancy (104).

Androgens and Biosynthesis of Prostaglandins and Other Eicosanoids

The impact of sex hormones on PG biosynthesis has been considered in various types of preclinical experimental animal models and in clinical studies, where sex differences in prostanoid formation were evident. In contrast to modulation of the 5-LOX pathway, the underlying mechanisms of the effects of androgens on PG formation are less clear and will be addressed here only briefly. Many studies reported on effects of androgens on PG formation in rodents with divergent and even opposing outcomes that seemingly depend on the experimental settings and conditions (**Table 2**). In fact, the first studies analyzing PG formation in the reproductive system of male rats in relation to androgens were conflicting: Bartke and Koerner documented reduced $PGF_2\alpha$ levels in the vas deferens, epididymis and the seminal vesicles of male rats upon castration which were reversed by administration of testosterone (20), while Sutherland et al. (21) found elevated $PGF_2\alpha$ in prostatic and vesicular glands upon orchidectomy of male rats which was reversed by testosterone application. It seems that androgens differentially influence PG biosynthetic pathways under physiological and pathophysiological conditions in various tissues and act at different levels. As a rule of thumb, androgens appear to elevate PG formation by augmenting COX-2 expression under healthy states, but they suppress COX-2 induction and PG biosynthesis under inflammatory conditions (**Table 2**). Thus, in the absence of an inflammatory stimulus, supplementation of testosterone in orchidectomized male rats increased COX-2 protein expression in cerebral blood vessels along with elevated PGE_2 levels (63, 64), in rat bladder epithelium (65), and PGE_2 and COX-2 were elevated in primary human coronary artery smooth muscle cells (HCASMC) by 5α-DHT (59). Conversely, in the presence of LPS or IL-1β, the increases in COX-2 protein in HCASMC (59) or in human monocytes (58) were attenuated by testosterone, which was observed also in LPS- or TNFα-stimulated HUVECs where the elevated mRNA levels of COX-2 were repressed when 5α-DHT was present (60). Also, under collagen-induced arthritis in castrated female rats, testosterone application possessed significant anti-inflammatory effects accompanied by reduced PGE_2 levels (62). While most of the PG-modulatory effects of androgens were connected to alterations in COX-2 levels, also the expression of mPGES-1 is seemingly affected by androgens. Thus, urinary PGE_2 levels and mPGES-1 expression in the renal inner medulla of spontaneously hypertensive rats was lower in males but after orchidectomy, PGE_2 metabolite excretion and mPGES-1 expression were elevated (75). Androgens may also affect the metabolism and transport of PG. For example, the PG-catabolic enzyme 15-PGDH and the PG-specific transporter OAT-PG are more abundant in the renal cortex of male rats with consequently lower renocortical PGE_2. Orchidectomization of male rats led to decreased OAT-PG expression and elevate renocortical PGE_2, and these changes were restored upon supplementation of testosterone (76).

Finally, androgens may impact the ω-hydroxylated AA metabolite 20-hydroxyeicosatetraenoic acid (20-HETE), generated by CYP enzymes, that mediates hypertension via multiple pathways. Several studies indicate that 20-HETE may play a role in androgen-induced vascular dysfunction and hypertension (105). Thus, treatment of normotensive mice with 5α-DHT induced both Cyp4a12 expression and 20-HETE levels in preglomerular microvessels, where 20-HETE can mediate both androgen-induced and androgen-independent hypertension (106). In an S6 mice strain, deficient in soluble guanylate cyclase-α1, hypertension was traced back to elevated Cyp4a12a expression and increased 20-HETE levels (107). Moreover, 20-HETE produced by CYP4a2 may contribute to elevated blood pressure in hyperandrogenic female rats (108).

CONCLUDING REMARKS

A variety of inflammatory diseases related to increased LT and PG are sex-biased with often higher incidence and severity in females, e.g., rheumatoid arthritis and asthma. Androgen-mediated sex differences in inflammation and in the activity of related innate immune cells are now well-documented. Androgens have a significant impact on neutrophils, monocytes and macrophages which are the major sources of inflammation-related LM that are biosynthesized on demand. Obvious sex differences in the capacity to generate LM led to the discovery of regulatory roles of androgens. For LT biosynthesis, the situation in males and females is rather unambiguous with superior LT levels in females, and there are clear indications for throughout suppressive effects of androgens on LT formation in neutrophils, monocytes and macrophages from human and/or mice (or rats). Androgens reduce LT biosynthesis in various inflammation models related to LT *in vivo* which can translate into impaired efficiency of anti-LTs. Intracellular signaling routes involving ERK-1/2 and/or PLD were uncovered as effectors of androgens in this respect which eventually prevents the assembly of the LT-biosynthetic 5-LOX/FLAP complex. Future studies will be needed to address by which receptor and signaling pathway androgens activate ERK-1/2 and how ERK-1/2 as well as PLD and DAGs are connected to aberrant 5-LOX subcellular localization preventing access to FLAP. Moreover, evaluation of the effects of androgens and other sex hormones on SPM formation is warranted in future research along with the regulation of related biosynthetic enzymes such as 12- and 15-LOXs. Sex specific differences in PG biosynthesis and the impact of androgens may depend on the organ/tissue but also on the presence or absence of inflammatory status: under healthy conditions, androgens may increase PG formation along with elevated COX-2 induction, while in presence of an inflammatory stimulus androgens may repress PG biosynthesis. Together, it appears that androgens significantly impact pro-inflammatory LM formation in innate

immune cells with direct consequences for pathophysiology but also for the pharmacotherapy of inflammation.

AUTHOR CONTRIBUTIONS

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REVIEW
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Influence of Androgens on Immunity to Self and Foreign: Effects on Immunity and Cancer

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It is well-known that sex hormones can directly and indirectly influence immune cell function. Different studies support a suppressive role of androgens on different components of the immune system by decreasing antibody production, T cell proliferation, NK cytotoxicity, and stimulating the production of anti-inflammatory cytokines. Androgen receptors have also been detected in many different cells of hematopoietic origin leading to direct effects of their ligands on the development and function of the immune system. The immunosuppressive properties of androgens could contribute to gender dimorphisms in autoimmune and infectious disease and thereby also hamper immune surveillance of tumors. Consistently, females generally are more prone to autoimmunity, while relatively less susceptible to infections, and have lower incidence and mortality of the majority of cancers compared to males. Some studies show that androgen deprivation therapy (ADT) can induce expansion of naïve T cells and increase T-cell responses. Emerging clinical data also reveal that ADT might enhance the efficacy of various immunotherapies including immune checkpoint blockade. In this review, we will discuss the potential role of androgens and their receptors in the immune responses in the context of different diseases. A particular focus will be on cancer, highlighting the effect of androgens on immune surveillance, tumor biology and on the efficacy of anti-cancer therapies including emerging immune therapies.

Keywords: androgens, immunity, cancer, immune cells, immunotherapy

INTRODUCTION

It has been known for a long time that sex is a biological variable directly affecting the immune response. Females are able to elicit stronger immune responses compared to males, leading to increased susceptibility to autoimmune diseases, while being less prone to infectious and malignant diseases (1). Sexual dimorphism in immunity has been attributed to a number of different factors, both endogenous, and environmental. Amongst the endogenous factors, one of the main contributors are sex hormones: estrogens and androgens (2). Androgens represent the male sex hormones, whose principal role is to trigger the development of male characteristics. They exert their biological functions through binding and activating the androgen receptor (AR)

(3). Several studies have shown that androgens/AR are involved in immunomodulation, thereby impacting innate and adaptive immunity. Altogether, they have been shown to induce different immunosuppressive effects: decreasing antibody production levels, lowering T cell numbers and activation capacity, and stimulating anti-inflammatory cytokine production by antigen presenting cells (4).

Many cancers entities are affected by activation of the androgen/AR signaling axis, resulting in more aggressive phenotypes which can be in some cases inhibited by androgen deprivation treatment (please see below) (5). Moreover, cancer is ultimately the result of failed immune surveillance. In this respect, immunosuppressive effects of androgens could dampen anticancer immunity and contribute to the male predominance apparent in most cancers (6).

In this review, we will discuss how androgens and the AR influence immune cells and cancer incidence and progression. Finally, we will discuss what is known about the impact of male sex and androgens on the efficacy of different immune therapies in mice and humans.

ANDROGENS

Steroid hormones are a group of cholesterol-derived hormones. They are produced by different tissues including the adrenal cortex, testes, ovaries, adipose tissue, breast, endometrium, prostate, skin, salivary gland, kidney, and by the placenta during pregnancy (7). Based on their receptors, steroid hormones are classified into five groups: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens.

The term "androgen" refers to any steroid hormone that has masculinizing effects (8). The biological actions of androgens, including testosterone and dihydrotestosterone (DHT) as well as androstenedione, dehydroepiandrosterone (DHEA) and its sulfated form (DHEA-S), are normally mediated through the androgen receptor (AR), a ligand-dependent nuclear transcription factor (9). After androgens are synthesized they are secreted into the blood stream predominantly as testosterone, which is mostly bound to sex hormone-binding globulin (SHBG). A very small fraction of testosterone (<3%) circulates as a free bioavailable form. Due to the high affinity of SHBG for testosterone, this globulin is regulating the amount of unbound testosterone available for target tissues (10). After entering its target cells, testosterone is converted to the most biological active form of androgens, dihydrotestosterone (DHT) by the enzyme 5α-reductase in most of the male reproductive organs. Testosterone can be also metabolized by aromatase into estradiol, primarily in fat tissues and in the hypothalamus, as well as in hematopoietic cells (11, 12). Therefore, local sex-hormone-mediated effects will be determined by the expression levels of either enzyme, as this will directly regulate the balance between androgen and estrogen production (13). This needs to be taken into account also in the context of androgen-mediated effects on immune cells (please see below). Careful experimental design is warranted in order to proof that observed phenotypes are directly caused by testosterone. Androgens, mainly testosterone and DHT, are the male sex hormones required for development of the male reproductive system and secondary sexual characteristics. In physiological conditions testosterone stimulates not only psychosexual behavior, but also physical and functional features. They include spermatogenesis, formation of the Wolffian duct, development of a deeper voice, bone mass, musculature, axillary, and pubic hair (3). DHT is responsible for the growth of the prostate and the external genitalia, as well as for male pattern of hair growth on the face and body and for male androgenic alopecia (12, 14). In summary, testosterone is more important in mediating anabolic effects while DHT is more potent in exerting androgenic effects.

ANDROGEN RECEPTOR MEDIATES ANDROGEN EFFECTS

Androgen Receptor Signaling

Most biological actions of androgens are mediated via the nuclear androgen receptor (AR). AR is a ligand-dependent nuclear transcription factor that belongs to the steroid hormone nuclear receptor family together with other members, including the estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR) (15).

There are two different pathways of androgen signaling, the canonical or genomic and the non-genomic or non-classical pathway (16, 17). Signal transduction through the classical AR happens in several steps. In the absence of androgens, AR is located exclusively in the cytoplasm and associated with heat-shock proteins (HSPs). Binding to the ligand induces the dissociation of AR and HSPs and leads the subsequent translocation of AR to the nucleus (18). Once AR is shuttled into the nucleus, ligand-activated AR binds specific DNA regulatory sequences [androgen response elements (ARES)] (19). This ligand-dependent transcription factor modulates gene expression through direct DNA binding and the recruitment of several coregulators to form complexes, which are necessary to induce epigenetic histone modifications and chromatin remodeling at target genetic loci (20, 21).

The activation of the non-genomic or non-classical pathway leads to rapid, transcription-independent effects of androgens caused by their binding to non-classical receptors including ZIP9 and GPRC6A (16, 17), which affect the regulation of other transcription factors, nuclear receptors and cytoplasmic signaling events. Non-classical receptors can also be associated to G-proteins in the plasma membrane (22, 23). Examples of effects induced by binding of androgens to non-classical receptors include activation of mitogen-activated kinase (MAPK), protein kinase C (PKC), protein kinase A (PKA), and increases in free intracellular calcium. In addition, AR can also be transactivated in presence of very low levels or absence of DHT via different cell surface receptors such as HER2. Signal activation emerges from different mechanisms, which are not necessarily mutually exclusive, including extracellular signaling peptides such as interleukin-6 (IL-6), epidermal growth factor (EGF),

and insulin-like growth factor (IFG). Altogether, the androgen-independent activation of AR occurs relatively often in cancer (22, 24).

The Role of Androgen Receptor in Health

In physiological conditions, the main role of the androgens/AR axis is the development of male characteristics, including spermatogenesis and the mediation of neurobiological and behavioral sex differences between female and male mammals already during the perinatal development (25). Behavioral gender dimorphism can be reflected in aggressiveness, parental care, or territorial behavior for example. Not only testosterone is necessary, but also estrogens are required during the early neonatal period for the development of male behavior traits. Here, testosterone is converted in estrogens through aromatase activity. This enzyme is expressed in brain cells including neurons. Interestingly, neurons expressing aromatase showed sex difference in their location within the brain. It was found that male mice had higher numbers of aromatase-positive neurons in the areas of the brain responsible for modulating aggressive and sexual behavior. In addition, it was demonstrated that estrogens are capable to masculinize aromatase positive neurons in these regions, contributing to the development of male behavior (26). In line with this, similar effects were observed in humans diagnosed with psychiatric syndromes, where males showed more aggressive behavior (27).

The AR is expressed in a diverse range of tissues and systems, besides the male reproductive organs. It can be found in muscle, bone, and adipose tissue, as well as in the immune, cardiovascular, neural, and hematopoietic systems, in which androgens have also been documented to exert biological actions (15, 28).

The bone represents the most important extragonadal site influenced by androgens/AR. In this regard, testosterone has important effects on bone physiology because when men are hypogonadal, a condition with too low levels of this hormone, bone homeostasis is severely perturbed. This perturbation results in osteopenia of regions richer in cortical bone, such as the radius, and in trabecular bone like the spine. These effects can be reversed upon replacement with testosterone (29). The effect of testosterone treatment on the bone of women with low serum levels of this hormone is not clear but probably small.

Another relevant extragonadal site of androgen influence is the cardiovascular system. Cardiovascular diseases are known to have significant sex disparity, with men presenting earlier onset and greater severity compared to women. Specifically, men have a 2 to 3-fold higher age-specific risk of cardiovascular death (30). Preclinical studies in a mouse model of pressure overload by transaortic constriction induced cardiac hypertrophy showed that treatment with a DHT conversion inhibitor, finasteride, reduced mortality in both sexes diminishing ventricular dilation and dysfunction, as well as pathological cardiac hypertrophy and fibrosis (31). Similarly, after orchiectomy in mice, detrimental cardiac remodeling and dysfunction generated by several stressors, was prevented with the removal of androgens (32). Altogether, increased androgen levels are important for cardiac pathophysiology.

Effects Androgen Receptor Mutations

Loss-of-function mutations are key players in the modulation of receptor functions. They can lead to changes in the structure of an encoded protein resulting in a decrease or complete loss of its expression. AR is located on the X chromosome and mutations are relatively common. In this context, androgen insensitivity is the most frequent form of genetic hormone resistance. Since males carry one copy of X chromosome, AR mutations with functional consequences are definitely expressed in all cells of affected males. In contrast, females bearing these mutations are silent carriers without any obvious phenotype because the functional allele on their second X chromosome will mostly counteract the effect. Nevertheless, there are some exceptions in which a small percentage of women (∼10%) carrying AR mutations exhibit mild phenotypic effects including mildly decreased body hair, delayed puberty onset, and/or increased height (33, 34). Due to the overall suppressive effect of androgens on lymphocytes (please see below), it would be very interesting to find out whether lymphocytes with an AR-mutated allele would be positively selected over cells carrying WT AR allele, as this is currently unknown. Further research is necessary to answer this relevant question.

Regarding the AR gene, many different types of mutations have been described. The most common comprise perturbation of the reading frame caused by insertions, deletions, splice site interruptions, and frame-shifts which often compromise protein function. Moreover, another typical mutation is single base replacement, whose effects can differ from no effect to a complete loss-of-function. In addition, other less frequent inactivation mechanisms induced by mutations exist such as for example loss of conformational stability resulting in inefficient or aberrant translation thereby diminishing the expression of functional AR protein.

Mutations of which the vast majority (more than 90%) are single base replacements occur at multiple loci within the AR gene. They have been shown to result in pathophysiological consequences when amino acid substitution takes place in the functionally crucial regions including the DNA-binding domain (DBD) or ligand-binding domain (LBD). However, if mutations occur in non-functional regions they can also represent silent polymorphisms (35). When AR mutations happen in the germline, the situation is similar, a broad spectrum of functional consequences can result ranging from absence of phenotypic changes to different androgen insensitivity syndromes (AIS). AIS exhibit different phenotypes correlated with the degree of impairment of AR function. The clinical consequences can be classified as complete, partial or mild. Complete androgen insensitivity syndrome in men (CAIS, previously known as testicular feminization) results in an undervirilized male phenotype with impaired differentiation of male gonadal tissue and incomplete development of the external or internal genitalia. Nevertheless, seemingly normal male phenotypes can also occur. Partial androgen insensitivity syndrome (PAIS) is characterized by impaired male genitalia development, showing external genital feminization and secondary sexual characteristics like breast development. The degree of conversion from male to female phenotype is correlated with the severity in which the

mutation functionally affects the AR. Mild androgen insensitivity (MAIS) is the least severe form of androgen insensitivity. It can be sufficient to minimally impair spermatogenesis, but these individuals display normal male genital differentiation, with only discreet changes in body habitus and size, as well as in face and body hair patterns (33, 36).

To corroborate the key role of AR mediating the biological effects of androgens genetic mouse models have been generated, in which the gene encoding AR has been knocked out (37–41). A phenotypic analysis performed in ARKO male mice showed that they have a female-like appearance and reduced body weight, compared to male wild-type (WT) mice. They have also about 80% smaller testes and lower concentrations of serum testosterone. Additional features include incomplete spermatogenesis, increased number and size of adipocytes, as well as reduced cancellous bone volumes compared with WT littermates. Moreover, in female ARKO mice, the average number of pups per litter is lower than in WT female mice, regardless of homo- or heterozygous genotype, pointing to possible defects in female ovulation and fertility (41).

Altogether, androgens and AR exert a central role in health and pathophysiology. Therefore, it is important to dissect the biological effects of this axis in different contexts including the immune system.

EFFECTS OF ANDROGENS ON THE IMMUNE SYSTEM

Beyond the roles described above for androgens/AR in regulating the male phenotype development, it has been demonstrated that they can also regulate immune function. AR can act directly on immune cells by influencing the transcription of immune-regulatory genes through DNA-binding-dependent and -independent mechanisms (21). Immune modulation exerted by androgens has been investigated in animal models and humans. These studies put forward androgens as important drivers of the well-described gender dimorphism in infectious and autoimmune diseases, with females being usually more susceptible to autoimmunity diseases, and less vulnerable to infections than males (42). In this context, it has to be taken into account that sex differences in immunity cannot be attributed solely to sex hormones but are multifactorial in origin and include effects due to X-chromosome inactivation and behavioral differences amongst others. It is beyond the scope of this review which is focused on androgens to discuss all factors potentially influencing the sex bias of the immune system and we refer the reader to recent comprehensive reviews in this field (1, 2, 43).

It was found that AR are expressed in a wide variety of innate and adaptive immune cells including neutrophils, macrophages, mast cells, monocytes, megakaryocytes, B cells, and T cells (44–52). Interestingly, AR are expressed also in hematopoietic stem cells and lymphoid and myeloid progenitor cells (44, 53, 54). For a comprehensive overview table of the different hematopoietic cell populations and their AR expression we refer the reader to a comprehensive review covering this topic (44). Therefore, androgens can directly influence both the progenitor and mature immune cell compartment.

Evidence derived from different studies points to a rather immunosuppressive role of androgens in different immune cell types mostly by reducing and/or promoting expression of pro-inflammatory and anti-inflammatory mediators, respectively [**Figure 1**; (55)]. In the following section, we will discuss what is known about the effects of androgens and AR in different innate and adaptive immune cells.

Neutrophils

Neutrophils, also coined polymorphonuclear (PMN) leukocytes, are the major cell type in human blood, and are considered the first line of defense in the innate immune system response. Their function is to identify and attack invasive microorganisms through phagocytosis and degrade the pathogens intracellularly. As consequence, granular material is released and neutrophils extracellular traps (NETs) are generated, helping to kill more pathogens (56).

AR is expressed in the majority of neutrophil lineages, including proliferative precursors like promyelocytes, myelocytes, and myeloblasts, as well as in mature neutrophils. AR expression patterns were not found to be differentially affected by gender in these cells (45). Androgens can promote neutrophil differentiation and recruitment, thereby increasing their numbers in mice and humans (44, 57, 58). Consistently, neutrophil numbers are decreased after castration and in ARKO- and Tfm mice, indicating that androgen and AR signals positively regulate neutrophil development. For example, ARKO mice have severe neutropenia with only one-tenth of the neutrophils of WT mice. Further analyses of the neutrophil lineage in ARKO mice showed that precursors and mature neutrophils are significantly reduced (59). In line with these findings, prostate cancer patients with drug-induced androgen blockade also display neutropenia (60, 61). Moreover, in addition to reduced neutrophil counts, functional defects of neutrophils were also observed in ARKO mice: neutrophils retain normal phagocytosis properties but respond less to granulocyte-colony stimulating factor-induced proliferation and to migratory signals *in vitro*. In addition, they are more susceptible to apoptosis and produce less proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokines (CCL2, CCL3, CCL4, CXCL1, CXCL4, and CXCL7) compared to neutrophils from WT mice (62). Altogether, these findings show that androgens/AR are important for neutrophil development and some important aspects of their functionality.

On the other hand, it was observed that testosterone can also foster the maintenance of immunosuppressive neutrophils pointing toward a novel mechanism of protection against autoimmune disease including the development of lupus-like disease in lupus-prone (NZB × NZW)F1 male mice. Here, Gr-1^{high}Ly-$6G^{+}CD11b^{+}$ myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature myeloid cells, displaying a neutrophilic nuclear morphology and immunoinhibitory action, were constitutively increased in male BWF1 mice compared to female mice, which was regulated by testosterone (63).

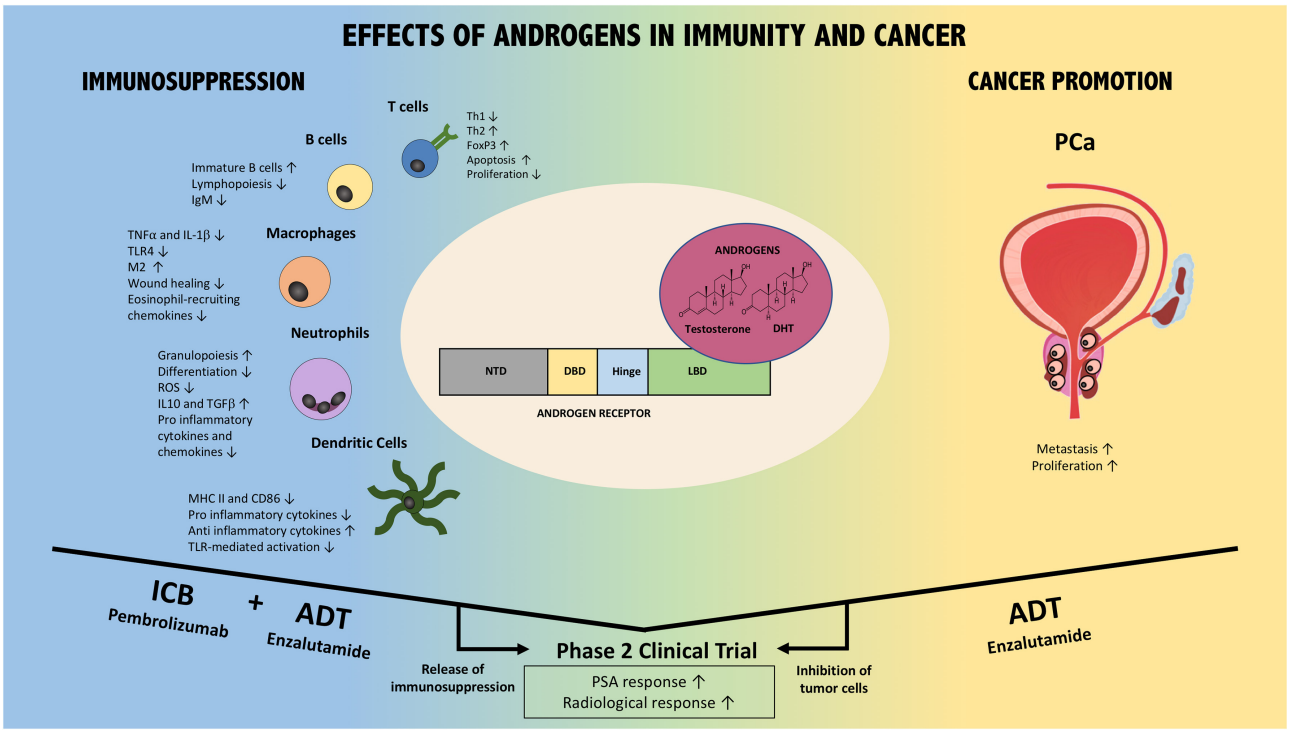

FIGURE 1 | Effects of AR/Androgen signaling in immunity and prostate cancer. AR/androgens can influence different immune cell subsets, including T cells, B cells, macrophages, neutrophils, and dendritic cells (Left part of the figure). Overall, their effect is immunosuppressive. In addition, androgens/AR directly and indirectly promote prostate cancer (PCa) via different mechanisms (Right part of the figure). Thus, the combination of ADT with immune checkpoint blockade could foster anti-tumor immune responses (ICB+ADT) while ADT additionally inhibits PCa directly. This combination strategy has resulted in improved patient responses compared to either monotherapy in Phase 2 clinical trials. Confirmatory Phase 3 trials are warranted and ongoing. NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand biding domain; DHT, dihydrotestosterone; ADT, androgen deprivation therapy; ICB, immune checkpoint blockade; PCa, prostate cancer; PSA, prostate specific antigen.

Furthermore, by using a bacterial model of prostate inflammation in male rats, it was shown that testosterone induces impaired myeloperoxidase and bactericidal activity in neutrophils. In addition to reduced functionality, an increase in the expression of the anti-inflammatory cytokines IL-10 and TGFβ1 was also observed, similar to what is observed in immunosuppressive "N2-like" neutrophils, which reside within the tumor microenvironment. These data reveal an interesting function of testosterone promoting inefficient and anti-inflammatory neutrophils leading to prolonged bacterial inflammation and an appropriate environment for several infectious diseases (57).

In summary, available literature indicates a dual and partly contradictory role of androgens/AR with a positive effect on neutrophil differentiation and some facets of their pro-inflammatory role while they can also support an immunosuppressive phenotype and inhibit bactericidal properties. Thus, more functional research and importantly investigations in humans are warranted to dissect the impact of androgens on neutrophils in health and disease.

Macrophages

Macrophages and monocytes, their precursors, are the "big eaters" of the immune system. They represent specialized cells involved in the detection, phagocytosis and destruction of bacteria and other foreign and harmful microorganisms. Macrophages are present in every tissue of the body. They originate from monocytes, which are quickly recruited upon infection or tissue damage, leading to their differentiation into tissue-specific macrophages. Their main function is to engulf pathogens or apoptotic cells and generate immune effector molecules. Moreover, macrophages are antigen presenting cells (APCs) that interact with T cells and initiate inflammatory response by releasing cytokines activating different populations of immune cells (64). Altogether, they play an important role in atherosclerosis, infections and wound healing. Both monocytes and macrophages were found to express AR, which was confirmed by functional studies (45, 47, 65).

The impact of androgens on macrophage function has been addressed in several studies and overall point to an (immuno)suppressive effect. For example, it was found that castrated male mice are significantly more susceptible to endotoxic shock, which results from a severe and generalized

pro-inflammatory response induced by systemic infection with gram-negative bacteria. Notably, this effect was reverted when the mice were treated with exogenous testosterone (66).

In line with this, a complex network of cytokine and immune cell interactions is present during sepsis in humans leading to high mortality due to organ dysfunction or failure. Macrophages are recognized to play essential roles in sepsis and influence both inflammatory responses and immune homeostasis. Besides, macrophage dysfunction is recognized as one of the main causes for sepsis-induced immunosuppression in mice and humans (67). Epidemiological studies identified male gender as an independent risk factor for the development of severe infection compared with females. Male sex hormones have been shown to have a suppressive effect on cell-mediated immune responses, including in macrophages. Consistent with this notion, it has been demonstrated in humans, that the female sex is protected from septic conditions requiring an active cell-mediated immune response, whereas the male sex has been associated to suffer deleterious consequences due to a reduced cell-mediated immune response, where splenic and peritoneal macrophage cytokine release are depressed. Remarkably, in preclinical models, administration of the anti-androgen flutamide after the induction of sepsis, was able not only to reestablish the low cytokine released levels by splenic macrophages and splenocytes, but also significantly decreased the mortality of post-hemorrhaged mice (68).

Rettew et al. demonstrated that testosterone could reduce the expression of Toll-like receptor 4 (TLR4) in a macrophage cell-line, in cultured primary macrophages and *in vivo* in mice (66). TLR4 is a transmembrane receptor that when activated leads to intracellular NF-κB signaling pathway induction and inflammatory cytokine production, promoting the activation of the innate immune system (69). However, more research is warranted to demonstrate a direct effect of androgens on the function and phenotype of macrophages.

Chronic inflammation induced by macrophages is strongly associated with cardiovascular disease. Inflammation is a key player in the development and progression of coronary heart disease (CHD) and testosterone has been shown to dampen the inflammatory response by suppressing the expression of TNF-α and IL-1β in stimulated human macrophages cultured *in vitro*. These results need functional validation in an *in vivo* setting, but lead to the hypothesis that testosterone could exert an anti-inflammatory effect on macrophages which could be explored in the CHD setting (70).

An unexpected role for androgen/AR was found in promoting M2 polarization of alveolar macrophages (AM), which correlates with asthma severity in humans. Asthmatic women present more M2 macrophages than asthmatic men, therefore androgens were used as an experimental asthma treatment. Using mice lacking AR specifically in monocytes/macrophages (AR^{flox}LysMCre), was observed only in males, and impaired M2 polarization leading to lung inflammation and reduced eosinophil recruitment, which could be due to a reduction in eosinophil-recruiting chemokines in alveolar macrophages deficient in AR (71).

On the other hand, castration of male mice or blockade of androgen action by flutamide hastened wound healing associated with lower macrophage infiltration, a dampened local inflammatory response and decreased expression of the proinflammatory cytokine TNF-α (72). This shows, that similar to the findings observed in neutrophils (please see above), androgens/AR mostly exert a negative influence on macrophage function, but can in certain conditions also promote their function.

Dendritic Cells

Dendritic cells (DCs) are APCs derived from bone marrow precursors and are widely distributed across the body. DCs are a heterogeneous group capable of initiating and orchestrating immune responses, acting often as messengers between the innate and the adaptive immune system. Their main function is to process and present antigens via MHC molecules to T cells. DCs exert immune-surveillance for exogenous and endogenous antigens and induce the activation of naive T cells, thus, orchestrating diverse immunological responses (73).

Overall, testosterone induces an inhibitory effect on DCs, nevertheless it remains unclear whether it is a direct or indirect effect because the expression of AR by DCs has not been clearly determined (44). In this context, there is one study performed in mice showing that bone marrow-derived DC (BMDCs) express ER, but not AR (74). Conversely, another study indicates that production of anti-inflammatory cytokines by BMDCs was increased at low to medium DHT exposure, suggesting the presence of AR. Additionally, in the same study carried out in mice, ChIP analysis was performed with tumor associated DCs, as well as splenic DCs revealing ERα and AR expression by DCs from both tissues (75). In addition, ER expression was found in hepatic DCs, suggesting altogether an influence of sex hormones on DC function in mice (76). However, the evidence is scarce at this point, especially concerning direct effects of androgens on DCs and further research is warranted in order to dissect these effects and clarify the role of estrogens.

Viral infections lead to different clinical manifestations between sexes in humans, and it has been reported that this is also the case for HIV-1 disease development. One of the differences observed is that during the response to Toll-like receptor 7 (TLR7) ligands, which are encoded by HIV, the production of interferon-alpha (IFN-α) by female plasmacytoid DCs (pDCs) is significantly higher than the levels produced by male pDCs. Accordingly, women develop more robust secondary activation of $CD8^+$ T cells. In line with these *in vitro* experiments, stronger $CD8^+$ T cell activation in women chronically infected with HIV-1 was observed compared to men, after normalizing the viral load for all the patients. These results point out that sex differences observed in the progression of HIV-1 may be due to stronger immune responses in women, which present higher activation of pDCs induced by TLR compared to men at a given viral load (77).

Sex differences in DCs have also been demonstrated using a well-studied mouse model of infection with lymphocytic choriomeningitis virus (LCMV). DCs isolated from brains of female mice with LCMV infection were considerably more activated, as shown by increased surface expression of MHC

class II and CD86 in female compared to male mice. Exogenous androgen administration to female mice or gonadectomy of male mice resulted in better response to the LMCV, however, neither resulted in the alteration of the DC population in terms of quantity or activation (78). Therefore, it is likely that in this case the immunomodulatory effects of androgens were not directly influencing the DC population.

A study of men with partial androgen deficiency showed that testosterone replacement led to decreased *ex vivo* production of proinflammatory cytokines (79). Another study was performed in men with hypogonadism, also known as testosterone deficiency, where the authors compared the distribution and functional status of peripheral blood (PB) monocytes and DCs ($CD16^+$) among other cell types compared to male control subjects. Interestingly, it was found that serum testosterone levels among hypogonadal men were negatively correlated with CpG (oligodeoxynucleotides)-stimulated expression of CD107b by $CD16^+$ DCs. These data suggest that low testosterone levels could enhance immune response by increasing circulating (activated) $CD16^+$ DCs (80). However, as mentioned previously, these findings represent a descriptive correlation and functional studies are necessary to investigate whether or not testosterone has direct effects on DCs. Here, it is of special importance that different hematopoietic and non-hematopoietic cell populations can express aromatase and can thus convert testosterone to estrogen. Hence, estrogen could also be responsible for observed effects exhibited by increased or decreased testosterone levels (13).

T Cells

T cells originate in the bone marrow, then migrate to the thymus for maturation and selection, and are subsequently exported to the periphery. They build an essential part of the immune system, coordinating multiple facets of adaptive immunity throughout life. The establishment and maintenance of homeostasis, specific, and memory immune responses depends on T cells. These cells present unique cell surface receptors that are created by randomly assorting V-, J-, C-, and D genes. These receptors recognize foreign particles (antigen) by a highly variable T cell receptor (TCR) expressed at the cell surface, allowing T cells to recognize and respond to diverse antigens derived from pathogens, tumors, and the environment. They also maintain immunological memory and self-tolerance and represent major drivers of many inflammatory and autoimmune diseases. Peripheral T cells comprise different subsets of cells. The thymus is the primary site of T cell differentiation into which T progenitor cells migrate from the bone marrow and undergo TCR rearrangement, giving rise to the two major subtypes of T cells: helper and regulatory ($CD4^+$) or cytotoxic ($CD8^+$) T cells (81).

The effect of androgens on T cells involves two major processes: thymic size and the differentiation of T helper cells. AR expression was detected in $CD3^+$, $CD4^+$, and $CD8^+$ thymocytes, with the highest expression in cytotoxic T cells (48, 49, 82).

It is well-known that androgen deprivation, due to castration or AR deficiency, causes enlargement of the thymus (83, 84). In this context, specific AR deficiency in T cells (T-ARKO mice) has no effect, while AR deficiency in thymic epithelial cells (TEC-ARKO mice) leads to decreased thymus size. Therefore, AR signaling exerts an indirect but potent effect of in T cell development (please see below) (85). In castrated mice, the administration of androgens completely reversed the thymic hypertrophy and significantly increased the total number of thymic cells expressing AR, nevertheless, it is unclear whether this effect is mediated through T cells (86, 87). Androgen treatment induced a rapid thymic involution suggesting a role for these hormones promoting apoptosis thereby influencing the size and composition of the thymus, as well as inhibiting T cell proliferation (84, 88). Testicular feminization mutation (Tfm) mice (C57BL/6J-ATa), which carry a defective AR gene, also show significant thymus enlargement, but in these mice androgen treatment failed to induce apoptosis in this organ. Notably, the apoptotic response to glucocorticoids was present, thus the apoptosis machinery was not compromised suggesting the requirement of a functional androgen receptor for the induction of androgen-induced apoptosis in the thymus (84). Moreover, the importance of the AR expression in thymic epithelial cells (TECs) as modulators of thymocyte development and its need for normal involutional response to androgens has been demonstrated using chimeric C57 mice, which were Tfm mice engrafted with WT bone marrow cells (89). Consistently, mice with specific deletion of AR in thymic epithelial cells (TEC) had an increase in thymic positive T cell selection, resulting in enlarged thymus and increased T cell numbers. Additionally, AR ablation in TECs enhanced bone marrow transplantation engraftment (85). Altogether, as androgens enhance thymocyte apoptosis, they could represent important mediators for thymocyte selection through direct signaling in TECs, and potentially transmit gender-specific features onto the peripheral T cell repertoire. In one study investigating the sexual dimorphism in central tolerance, was demonstrated the importance of AIRE (autoimmune regulator), which is differentially expressed between the sexes in mice. Results showed that murine female TECs express less AIRE compared to TECs from male mice. The role of androgens was confirmed by orchiectomy, where the lack of male hormones phenocopied female AIRE expression levels. Using an AIRE deficient *in vivo* mouse model, a link between sex biased AIRE expression and increased susceptibility of males to experimental autoimmune thyroiditis (EAT) was established (90). In another autoimmune disease study, a similar sex biased effect of AIRE expression in medullar TECs (mTECs) was observed. Here, the enhanced expression of AIRE by androgens in males was correlated with a protective role in an experimental autoimmune encephalitis (EAE) mouse model (91).

Alterations in circulating levels of gonadal steroids not only affect thymus size, but also affect thymic egression of T cells. In one study carried out in a cohort of healthy vs. hypogonadal men before and after testosterone replacement therapy it was observed that hypogonadism is linked with elevated thymic output of T cells. Consistently, this increase in peripheral T cells was reversed by androgen replacement (87). Furthermore, castration of post-pubertal male mice indicated that T cell numbers in peripheral lymphoid tissues are augmented upon androgen deprivation. In addition, T cells isolated from these

castrated mice proliferate more actively in response to TCR- and CD28-mediated co-stimulation as well as to antigen-specific activation compared to the same cells isolated from sham mice (92). Thus, androgens inhibit the number and the receptor repertoire of thymic T cells entering the periphery. Similar findings have been obtained in humans with prostate cancer. ADT resulted in an increase of circulating naïve T cells and of Th1-biased phenotypes. In studies using short-term ADT before prostatectomy an increase in oligoclonal T-cell infiltration into prostate tissue was observed (93). However, a study in prostate cancer patients undergoing androgen deprivation therapy showed a correlation of lower testosterone levels with lower $CD4^+$ and $CD8^+$ T cell counts at all studied time points (94). The reasons for this discrepancy remain unclear at present but it has to be taken into account that androgen deprivation therapy can also inhibit T cells via off-target effects (please see below).

Androgens not only influence the numbers of peripheral T cells but also affect their responses. Thymocytes and lymphocytes isolated from female mice react more effectively than male cells in mixed lymphocyte reactions (MLR). It was also observed that the production of interleukin 2 (IL-2) was higher in stimulated spleen cells from female mice compared to male or female cells treated with testosterone. In addition, castrated male mice showed increased while androgen-treated female mice exhibited decreased efficacy of antigen presentation assessed by increased lymphocyte proliferation in MLR (95). Altogether, these results confirm the suppressive role of androgens at the level of T cell activation.

T helper (Th) cells polarization arises after T cell receptor (TCR) of $CD4^+$ T cells interact with the antigen presented by the major histocompatibility (MHC) complex of professional APC. T helper cells (Th0) mainly differentiate into two functional subtypes, Th1 and Th2. Th1 are pro-inflammatory and characterized by the expression of IFN-γ, IL-2, and TNF-α, while Th2 are anti-inflammatory and express IL-4, IL-5, IL-10, and IL-13 (96). Murine Th cells from males tend to have a more pronounced Th2 cytokine profile, while their female counterparts express more Th1 cytokines. Consistently, androgen treatment enhanced production of IL-10 by murine $CD4^+$ T cells, thereby creating a shift toward Th2 responses (97). Additionally, androgens exert an overall inhibitory effect on Th1 differentiation by reducing the phosphorylation of STAT4 mediated by IL-12 (98). Moreover, in an induced mouse model of Grave's disease (an autoimmune disorder that results in hyperthyroidism and is caused by autoreactive T cells killing thyroid cells) (99), mice that were treated with DHT before disease induction had significantly lower IFN-γ and IL-2 production, consistent with an immunosuppressive effect of DHT on $CD4^+$ Th1 T cells (100).

Taken together, these findings may explain the lower incidence of autoimmune disease as well as the increased tendency to viral infections in males.

Treg Cells

Regulatory T-cells (Treg cells) are one of the most versatile immunosuppressive cell population and act as immunological sentinels in different tissues. Lack of Tregs in male mice and men lead to immune tolerance failure and autoimmunity in different organs. Consistently, Treg show continued AR expression after differentiation (50, 101).

Fijak et al. demonstrated that *in vitro* testosterone treatment of naive T cells resulted in an expansion of rat murine Treg cells with immunosuppressive activity. Moreover, in the same study it was observed that substituted testosterone levels in experimental autoimmune orchitis (EAO) in rats significantly increased the number of Treg cells ($CD4^+CD25^+Foxp3^+$) compared with EAO control animals (102). The same effect was observed *in vivo*, in a systemic lupus erythematosus mouse model, where DHEA administration restored normal levels of Tregs (103). The transcription factor Foxp3 represents a master regulator of Treg function. Interestingly, it was shown that FoxP3 expression can be modulated by testosterone due to direct AR binding to FOXP3 gene regulatory sequences, which could be directly responsible for the increased number of $CD4^+CD25^+Foxp3^+$ Treg cells upon testosterone treatment (50). Consistently, in a human study it was shown that in medically castrated men, where testosterone levels were reduced, deficiencies in number and function of Tregs were found (104). These results may lead to better understanding and treatment of autoimmune diseases.

A recent *in vivo* murine study assessed whether the function of immunosuppressive Treg cells is responsible for sexual dimorphism in visceral adipose tissue (VAT). VAT represents a hormonally active fat tissue localized around the internal organs. An immunophenotyping screening of VAT tissue showed that males have significantly higher number of Tregs compared to their female counterparts. Next, RNA-seq analysis from male Treg cells isolated from either VAT or spleen tissue showed significant differences in the transcriptional profile involving important regulators of the immune system such as Klrg1, Ccr2, IL-10, or Gata3, between these tissues. Notably, this effect was not observed in female Tregs isolated from the same tissues. Interestingly, this male-specific effect was regulated by androgens through increased numbers of IL-33 producing stromal cells because IL-33 leads to recruitment and expansion of Tregs cells in VAT (105).

B Cells

B cells form the center of the adaptive humoral immune system and are responsible for the production of antigen-specific immunoglobulins (Ig), commonly known as antibodies. Antibodies are directed against and can clear invasive pathogens (106). Early B cell development occurs in the fetal liver prenatally, before continuing in the bone marrow throughout life.

It is well-described, that regardless of age, females display in general higher numbers of B cells and basal immunoglobulin levels, resulting in greater antibody responses than males. Based on these facts, antibody responses to bacterial infections and viral vaccines are often stronger in females than males, thereby influencing susceptibility of males and females to various malignancies, autoimmune, and infectious diseases (1). Estrogens have been associated with a higher prevalence of autoimmune diseases in females, in which the role of B cells involves different cellular functions, including secretion

of autoantibodies, autoantigen presentation, and secretion of inflammatory cytokines (107).

In order to better understand the mechanism of the stronger immune response in females, Furman et al. analyzed the antibody response to a trivalent inactivated seasonal influenza vaccine (TIV) in 37 male and 54 female human subjects. The results showed higher secretion of inflammatory cytokines which could be responsible for enhanced antibody response to TIV in the plasma of women compared to men. Interestingly, a correlation was found between a cluster of genes involved in lipid metabolism, testosterone levels and the response to TIV in men. Thus, those men with higher testosterone levels showed increased expression of these genes accompanied by a poor response to vaccination. This effect was not seen in men with low levels of testosterone or women (108).

Another recent study carried out in mice, showed a sex biased B cell positioning in germinal centers (GCs) representing regions inside the secondary lymphoid organs where B cells can proliferate and mature. The fact, that female B cells are more efficiently positioned within GCs can result in a stronger humoral immune response and also enhance the prevalence to autoimmune diseases displayed in females. The binding of CCL-21 (chemokine ligand 21) to GPR174 receptor expressed on B cells leads to increased male biased migration of B cells toward the periphery of GCs. Consistently, castrated male mice showed a defective GPR174/CCL-21 driven migration, and this effect was rescued upon testosterone replacement. In the same way, female mice supplemented with testosterone mimicked male B cells migration patterns, indicating a sex biased androgen mediated mechanism in B cell immunity (109).

It was reported that the absence of AR expression in B cells, regardless of mouse strain, as well as in castrated WT mice, resulted in an elevated number of B cells in blood and bone marrow (52, 110). Similarly, castrated male mice exhibited higher number of fibroblastic reticular cells expressing BAFF, an essential factor for the survival of B cells. Consistently, the blockade of BAFF receptor by an antibody in male mice phenocopied the enhanced B cell numbers induced by castration. Interestingly, assessment of serum BAFF levels in healthy men showed a correlation of high levels of this cytokine with low levels of testosterone indicating translational relevance of this immunosuppressive mechanism (111).

It is known that AR is expressed in B cell progenitors but not in mature or peripheral B cells, therefore they are sensitive to androgens primarily during development (112). Thus, physiologic levels of androgens regulate in part the production of B lymphocytes, and increased B cell numbers occur in conditions when androgen levels are decreased.

The effect of androgens/AR on B lymphocytes was further confirmed by Altuwajri et al., who observed higher levels of immature B cell development in G-ARKO (global AR knockout) mice comparable to observations in Tfm mice, and also in castrated BALB/c mice. DHT pellets implantation restored the normal B cells levels in bone marrow of castrated mice but not in G-ARKO mice, supporting the hypothesis that androgen-mediated B cell maturation is AR dependent (110). In another study, conditioned medium generated from DHT-treated bone marrow derived cells (BMDCs) resulted in inhibited B cell colony formation. However, this capability was not altered when the conditioned medium was harvested from DHT-treated BMDCs of Tfm mice, proposing an important role for AR in BMDCs mediating the observed differences of B-cell numbers (113).

Furthermore, castration has been shown to significantly increase spleen weight, as well as the total number of peripheral blood B lymphocytes. The increase in circulating B cells was largely due higher numbers of B cell progenitors in the bone marrow with a B220(lo^+) CD24(hi^+) phenotype, and this increase was sustained in castrated mice for at least 54 days. After quantifying B cell progenitors in the bone marrow, it was observed that relative numbers of these cells responding to IL-7, including early pro-B cells, late pro-B cells, pre-B cells and immature B cells, were significantly raised. Therefore, androgen deprivation mainly augments numbers of IL-7-responsive B cell progenitors (114).

It is well-described that puberty represents the peak at which sex steroids influence the difference between sexes. However, the impact of sex hormones can begin as early as *in utero*, which could lead to sex disparities in different immune cell populations very early in life. DHT actions *in utero* could influence already peripheral B-cell maturation due to the higher levels of this hormone among boys in the cord blood. In this context, there is one study showing different proportions of immature $CD5^+$ B cells between boys and girls already at the age from 3 to 8 years. In one study, testosterone and DHT levels were measured in blood samples obtained at birth and at 8 years of age. Here, a positive correlation between DHT levels at birth and higher proportions of $CD5^+$ and immature B cells indicating delayed B cell maturation was found in 8-year-old boys (115).

Together, these findings illustrate the importance of androgen/AR in B cell homeostasis, pointing to the fact that androgens inhibit B lymphopoiesis.

THE EFFECTS OF ANDROGENS IN CANCER

In cancer the immune system fails to mount an adequate response to combat malignant cells (55), therefore cancer is also the result of failed immune surveillance amongst other causes. There are several studies showing that cancer incidence and mortality is higher in males compared to females, with the exception of few entities including thyroid and gallbladder cancer (2). As we described in the previous section, androgens affect the number, and function of different immune cells (**Figure 1**). Next, we will review the actions of androgens and AR in prostate cancer (**Figure 1**). We will focus on this malignancy because in prostate cancer the role of male hormones and its receptors have been the most extensively studied and have the highest clinical relevance. Due to this fact, a substantial number of inhibitors were developed in order to treat prostate cancer. The aim of these therapies is to decrease male hormone levels and AR signaling activation, since this axis is promoting tumor progression. Different androgen deprivation drugs exist and they can be classified in two main

classes depending at which level androgen/AR signaling is blocked (116, 117).

The first group comprises luteinizing hormone-releasing hormone (LHRH, produced by the pituitary gland) agonists and antagonists, which were amongst the first therapies developed to reduce the amount of testosterone produced by the testicles. GnRH agonists induce an initial massive gonadotropin secretion, which causes the pituitary gland to become desensitized and consequently leading to dramatic suppression of LH. In contrast, GnRH antagonists directly suppress the receptor by competitive inhibition of LH. The LHRH agonist group comprises the following approved drugs: leuprorelin, buserelin, triptorelin, and goserelin. The only LHRH antagonist approved for the treatment of prostate cancer is degarelix [**Figure 2**; (116)].

The second group are coined anti-androgens because they inhibit androgen synthesis and/or block the binding between androgens and AR. Approved anti-androgens that target androgen synthesis are: abiraterone (which inhibits 17α-hydroxylase), finasteride and dutasteride (which block 5α-reductase action). The first generation of anti-androgens that block the binding of male hormones to their receptor, or inhibit AR nuclear translocation are: bicalutamide, flutamide, nilutamide, and cyproterone (the only steroidal one). A second generation of anti-androgens was synthesized, which have a similar mode of action as the first generation but show improved potency and efficacy. In addition to the above-mentioned mechanisms, these drugs inhibit AR DNA binding and recruitment of co-activators. They include: enzalutamide, apalutamide, and darolutamide. Finally, galeteronel is the only compound with dual androgen antagonist and biosynthesis inhibitor function, but is still pending approval [**Figure 2**; (116, 118)].

For a more extensive description of the role of androgens/AR in human cancer besides prostate cancer we refer the reader to comprehensive reviews of this field (119).

Prostate Cancer

The most well-known and -studied androgen/AR-dependent cancer is prostate cancer. With nearly one in every seven men diagnosed during their lifetime, this cancer is the second most frequent in men. Since Huggins and Hodges proved the dependence of prostate cancer on AR pathway by androgen deprivation, it became obvious that this cancer relies on androgen/AR signaling for proliferation and survival [**Figure 1**; (120)]. This was confirmed by whole genome association analysis (WGA), demonstrating that those genetic

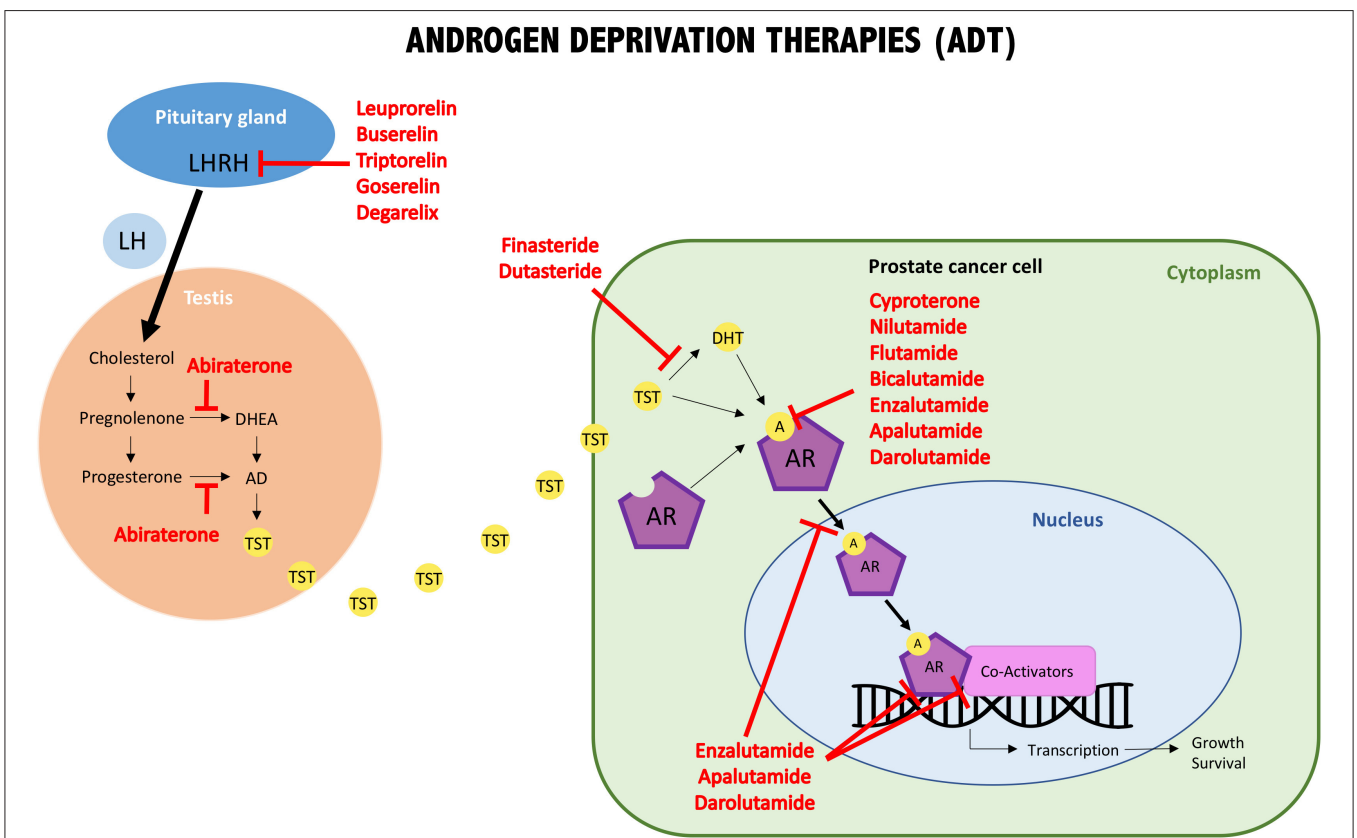

FIGURE 2 | Schematic illustration of androgens/AR interaction, intracellular pathway, and molecular targets of androgen deprivation therapies (ADT). One important group of ADT drugs are the LHRH analogs. They reduce the release of LH, which promotes TST production by the testis. The other important groups are the anti-androgens, either involved in blocking androgens at the synthesis level or involved in interfering with androgen/AR binding (AR blockers). LH, luteinizing hormone; LHRH, LH releasing hormone; DHEA, dehydroepiandrosterone; AD, androstenedione; TST, testosterone; DHT, dihydrotestosterone; A, androgens; AR, androgen receptor.

loci identified as potential prostate malignancy promoters contain an accumulation of AR- or AR-coactivator binding sites (121). This finding was corroborated by a study of germline mutations, which identified one mutation in the homeobox transcription factor (HOXB13) known to interact with AR, leading to a 20-fold increased risk of inherited prostate cancer (122, 123).

Notably, the link between testosterone and prostate cancer risk is ambiguous at present. For example, some studies revealed a higher risk of prostate cancer among men with low testosterone levels compared to those men with higher levels (124). However, the link between prostate cancer risk and circulating DHT, testosterone or other sex steroids could not be established, even though many population-based studies with this topic have been performed (125).

For example, a meta-analysis published in 2016 found no relationship between testosterone levels in men and their risk of developing prostate cancer, indicating that prostate cancer risk could be unrelated to endogenous testosterone levels (126). Another meta-analysis showed that testosterone therapy did not increase the risk of prostate cancer nor led to its progression in men who have already been diagnosed (127). Furthermore, testosterone replacement therapy also did not increase the levels of prostate specific antigen (PSA), a protein that is elevated in the bloodstream of men with prostate cancer (128).

In some observational studies, more aggressive prostate cancers have even been linked to lower testosterone levels. However, the extrapolation of information from population-based studies is hampered by the fact that many risk factors for prostate cancer, such as obesity, age and associated insulin excess, are correlated with declines in circulating testosterone (125). For this reason, the connection between the serum levels of testosterone and prostate cancer prognosis can differ depending on the clinical settings (129). Altogether, assessment of testosterone levels in circulation have failed to accurately relate prostate cancer incidence or prognosis.

Many pre-clinical and clinical studies have highlighted the importance of AR in prostate cancer. Mouse xenograft models demonstrate that AR^{+} castration-resistant prostate cancer (CRPC) is sensitive to enzalutamide, an AR inhibitor that competitively inhibits androgen binding to the receptor and consequently inhibit AR nuclear translocation and interaction with DNA but $AR^{-/low}$ CRPC is resistant. Consistently, *in vitro* data have shown that genome editing-derived AR^{+} LNCaP cells are sensitive while AR-knockout cells are resistant to enzalutamide (130). Another *in vivo* study has dissected the function of AR in prostate stromal and epithelial cells. To achieve this aim two mouse models were generated: inducible-(ind)ARKO-TRAMP, in which the AR was knocked down in both cell types, and prostate epithelial-specific ARKO TRAMP (pes-ARKO-TRAMP), in which the AR was knocked down only in the prostate epithelium. Findings in both mouse models indicate that the lack of AR leads to less differentiated primary tumors. Interestingly, the results obtained at initial stages in ind-ARKO-TRAMP mice showed less proliferative prostate tumors with smaller size while tumors generated in pes-ARKO-TRAMP mice were proliferating faster and thus larger prostate tumors were present. These data indicated that an early stage of tumor development, the main player involved in primary tumor growth is the prostate stromal AR rather than the epithelial AR. The possible dual roles of androgen action may require reevaluation of ADT regimens, regarding target, and timing in the treatment of prostate cancer patients. These results underline the necessity to develop new selective drugs to specifically target stromal AR in prostate tumors, at least at early stages (131).

Unfortunately, androgen deprivation therapy (ADT) ultimately leads to resistance development in prostate cancer patients. Castration resistant prostate cancer (CRPC) becomes evident after a median of 18–24 months of ADT. In this stage cancer cells are able to proliferate independent of testosterone mostly through androgen-independent AR signaling. Even after ADT, when testosterone is almost not present in the serum of the patients, the AR pathway activation is maintained by different mechanisms, such as upregulation of AR expression, production of androgens outside of the gonads including within the tumor tissue itself, induction of AR mutations leading to ligand independent activation and changes in the coregulator profiles (132).

THE ROLE OF ANDROGENS IN ANTI-CANCER IMMUNE THERAPY

The immune system plays an important role in tumor biology because it can influence essential steps of tumor development like growth, invasion, and metastasis. Tumor cells employ different mechanisms to evade immune elimination, which include: loss of antigenicity, loss of immunogenicity, and orchestration of an immune suppressive microenvironment (133). For this reason, cancer progression occurs in the context of failed immunosurveillance and therefore, strategies designed to harness the natural abilities of the immune system have become very promising approaches to treat cancer.

The principal strategy of cancer immunotherapy is to (re-)boost the immune system against tumor cells in order to allow clearance of malignant cells through mono- or combination treatments. Many different types of cancer immunotherapies exist and are divided in two groups according to their passive or active nature. Passive immunotherapies comprise monoclonal antibodies that target tumor specific antigens. Active immunotherapies represent the larger group including: cytokines/adjuvants, immune checkpoint inhibitors, adoptive CAR T cell transfer, and therapeutic cancer vaccines (134). Due to the outlined overall inhibitory effect of androgens/AR on immune cells it is likely that they also influence the response to anti-cancer immune therapies. In the next section we will overview what is known concerning these hormones and sex-biased differences in the response to immune therapies in mice and humans. Furthermore, preclinical and clinical data of combined ADT and immune therapies are reviewed with a focus on prostate cancer.

Preclinical Data

Nowadays, immune checkpoint blockade has become one of the most promising cancer immunotherapies. In the preclinical setting, there are no studies yet documenting a direct role

of androgens on responses to immune checkpoint inhibitors. However, sex biased response to PD-L1 blockade was observed by Lin et al. In this study, they showed in a preclinical model, in which mice were injected with B16F10 melanoma cells that anti-PD-L1 treatment significantly reduced tumor growth in female compared to male mice. This effect could be partially explained by the inhibited Treg function upon PD-L1 blockade in female mice and enhanced immune response compared to male mice (135). This study suggests that androgens present in males could have a negative impact on the response to anti-PD-L1 treatment—although other underlying mechanisms are also possible.

Type 2 innate lymphoid cells (ILC2) originate from common lymphoid progenitors. They play a crucial role in regulating type 2 inflammation in response to infections with parasites and can promote allergic processes. ILC2 cells are located in different mucosal tissues, like lung, or colon for example, but also in other tissues including liver, fat tissue, and bone marrow among others (136). In these cells, expression of AR has been reported and interestingly the frequency of ILC2 cells in the lungs is sex biased, with higher numbers in females compared to males. Consistently, in castrated mice this cell population in lungs was present in higher numbers compared to male mice. This enhanced presence of ILC2 cells could be one reason for to the enhanced susceptibility of women to develop asthma (137, 138). In the context of cancer, it has been published recently that ILC2 cells can infiltrate human and mouse pancreatic adenocarcinomas and are then designated TILC2 cells. Interestingly, TILC2 cells express the PD-1 receptor. Using a pancreatic mouse model, it was observed that mice treated with anti-PD-1 antibodies exhibited an increased number of TILC2 cells. These cells enhanced tissue-specific tumor immunity by priming $CD8^+$ cells and recruiting DCs. Moreover, augmented TILC2 frequencies were associated with longer survival of mice (139). This important study identified activated TILC2 cells as a target of anti-PD-1 immunotherapy and due to their sex biased number in lungs it will be of interest to determine if these cells are involved in mediating sex differences in response to ICI.

Adoptive T cell transfer represents another attractive novel option in cancer treatment. Interestingly, one in *vitro study* indicated an additive effect of ADT and CAR-T cells. Here, T cells were engineered to recognize the aberrantly expressed prostate tumor protein Muc1 and were subsequently able to specifically lyse PC3 cells. Moreover, the combination with the anti-androgen flutamide, was feasible and led to additive anti-tumor effects compared to either therapy alone (140).

Other immune therapies such as therapeutic vaccines have been studied in combination with androgen ablation leading to interesting findings. For instance, in a spontaneous prostate cancer mouse model (TRAMP), a yeast-based vaccine expressing Twist antigen (present in metastatic cells which underwent EMT) was combined with the AR antagonist enzalutamide, resulting in improved survival compared to either monotherapy or untreated control group (141). Another study in a prostate cancer mouse model showed that castration, although not sufficient to prevent invasive and resistant tumor growth, elicited enhanced T cell numbers within the prostate tumors as well as a higher $CD8^+/Foxp3^+$ T cell ratio. Anti-CD25 was used to induce additional Treg depletion, but proved to be insufficient as monotherapy in terms of immunostimulation. For this reason, a second therapy was added which was based on intraprostatic injection of tumor cells expressing an antigen called LIGHT, which is able to recruit and activate T cells to the tumor site, causing rejection of antigenically unrelated tumors. Results showed that combination of castration with both anti-CD25 and LIGHT cell vaccine was more effective in reducing tumor burden and preventing tumor recurrence, compared to castration plus either monotherapy. This improved efficacy was due to immune modulations preventing Treg accumulation and augmentation of effector cells infiltrating the prostate epithelium (142).

Nevertheless, the relationship between androgen deprivation and immunization is not always straightforward. In a mouse study of prostate cancer therapy with ADT and DNA vaccination, *ex vivo* analysis of isolated DCs from the spleens and lymph nodes of castrated and sham-castrated mice showed that simultaneous androgen deprivation increased DCs numbers, but did not improve their costimulatory function for cytotoxic T cells. However, if castration was performed after immunization, androgen ablation was able to increase the immune response elicited by vaccination resulting in increased DC function and T cell cytotoxicity (143).

From a slightly different perspective, a study by Olson et al. showed that prostate cancer cells express higher levels of AR upon androgen deprivation, which in turn improves recognition of tumor cells by AR-specific T cells (144, 145). Therefore, direct targeting of the AR could be a promising immunotherapeutic approach. In line with this hypothesis, immunization of HHDII-DR1 mice, which express human HLA-A2 and HLA-DR1, with a DNA vaccine encoding the androgen receptor, pTVG-AR, augmented HLA-A2-restricted immune responses. This led to lysis of syngeneic prostate tumor cells, resulting in a reduction of tumor burden concomitantly with an improved overall survival of tumor-bearing mice (146).

Even though most studies focusing on androgen ablation in combination with cancer immunotherapy have been performed in prostate cancer for obvious reasons, a study by Hsueh et al., found that androgen blockade enhances the response to a melanoma vaccine in a syngeneic murine model. Here, the combination of flutamide treatment followed by irradiated cell vaccine, prior to melanoma inoculation, resulted in better survival rate compared to either flutamide or vaccine alone, as well as to the untreated group (147).

Altogether, preclinical data suggest that androgen deprivation therapy (surgical and medical) could potentially be used in combination with different kinds of immunotherapies. However, it is important to note that there is a caveat regarding certain medical ADT therapies. In a study published by Pu et al., it was observed that orchidectomy in combination with CpG vaccine was beneficial in terms of survival and immune response in a murine model of prostate cancer. However, some AR antagonists (flutamide and enzalutamide) showed unexpected immunosuppressive effects when given in combination with the same vaccine. This immunosuppressive effect was likely due to an elusive off-target effect on T cells, leading to impaired

activation. This led to ineffective immunization when given simultaneously but not when applied before ADT. Notably, the use of alternative ADT therapies such as androgen biosynthesis inhibitors in combination with CpG vaccine showed success in synergistic inhibitory effects on cancer tumor growth in mice (148). These findings illustrate the importance of meticulous preclinical research in order to optimize combination partners and timing of combined immuno- and ADT, which should inform the design of rational clinical trials.

Clinical Data

Immune checkpoint inhibitors (ICIs) lead to promising outcomes in some but not all cancer entities. Because of the importance of this treatment modality the following section will focus on what is known concerning the influence of male sex (and therefore possibly androgens) on the therapeutic response to ICIs in humans.

ICIs mostly withdraw inhibitory signals of T-cell activation, thereby tumor-reactive T cells are able to surpass negative regulatory mechanisms and exert a more potent antitumor immune response (149). Currently, monoclonal antibodies targeting T-lymphocyte-associated antigen-4 (CTLA-4), programmed-death 1 and programmed death-ligand 1 (PD-1 and PD-L1) represent key ICIs. They have been already approved for certain entities including lung-, bladder-, kidney-, skin-, and head-and-neck cancer amongst others (150, 151). Today, many phase I-III clinical trials are being carried out worldwide to evaluate the efficacy of multiple ICIs as mono- or combination therapy for many different cancers (150).

Despite encouraging findings, low response rates were observed in some tumors. For example, treatment with ICIs in melanoma and non-small cell lung cancer has relatively high efficacy while in other entities such as pancreatic cancer, breast cancer and many sarcoma entities, response rates remain low. Moreover, only a relatively small part of cancer patients experience long-term benefit from ICI treatment and a significant number of patients experiences immune-related adverse events during therapy. This is not too surprising because inhibition of immune checkpoints can cause autoimmune responses to healthy tissues (152, 153). Thus, there is a need to develop predictive biomarkers in order to differentiate responding and non-responding patients, to reduce adverse effects and possibly anticipate the requirement of combination therapy for patients unlikely to respond to ICIs (154).

As described in the first section of this review, it is known that sex is a variable affecting both innate and adaptive immune responses (1). Nevertheless, it is alarming that <10% of cancer immune therapy-related data are analyzed taking into account the sex of the animal or human subjects (155). This is even more concerning because the available meta-analyses of large ICI trials in different entities suggest that ICIs could show different efficacy according to the sex of cancer patients, pointing to better results in males than females (151). However, the sex bias in response to anti-cancer immune therapies is an ongoing matter of debate and could not yet be resolved.

One important meta-analysis in this context has been carried out by Conforti and colleagues. The authors assessed the difference in ICI efficacy between men and women from 20 randomized controlled trials of ICIs (ipilimumab, tremelimumab, nivolumab, or pembrolizumab) including more than 11.000 patients showing overall survival according to the sex of the patients. The study contained patients with different advanced or metastatic cancers (67% men and 33% women) and the most common cancer entities were melanoma (32%) and NSCLC (31%). This analysis revealed a higher reduction in the risk of death in males compared to females upon treatment with the different ICIs. Most importantly, it was reported that overall survival was improved by these therapies for all patients, but that the magnitude of benefit is sex-dependent (156).

However, in a different meta-analysis of 23 randomized clinical trials, in which ICIs were used, 7 more additional clinical trials were included compared to the meta-analysis described before (156). In this study more than 13.000 patients were analyzed of whom 68% were men and 32% women. An overall survival benefit upon treatment with ICIs alone was found for both men and women with advanced solid malignant neoplasm (48% NSCLC, 17% melanoma, 9% renal cell carcinoma, 9% SCLC, 4% urothelial, gastric, head, and neck squamous carcinoma and mesothelioma). In this analysis, no statistically significant differences between the sexes were observed (151).

After showing that men obtained larger benefit than woman from therapy with anti-CTLA-4 or anti-PD-1 agents, Conforti et al. performed a second study where the authors investigate whether the combination of chemotherapy and anti-PD-1 or anti-PD-L1 could be more effective in woman compared to men. The meta-analyses were conducted with data from 11 randomized controlled trials comparing progression free survival (PFS) and overall survival (OS) in patients who received combination of ICI therapy with chemotherapy with those who were treated with ICIs or chemotherapy alone. In this study, the results concluded that women had better responses to the combination of ICI and chemotherapy compared with men, while men responded better to either chemotherapy or ICI therapy alone compared with women (157). A recent paper published in by Ye et al. addresses the issues regarding the conflicting results generated by meta-analyses regarding sex differences in response to ICB. They point to the fact that due to the substantial heterogeneity of the clinical trials included, especially considering the control arms, a meta-analysis approach was not the proper analysis to be performed on these data sets as a whole, as effects could have been masked or diluted. As a result, they decided to take a different approach and used ICB treatment data sets with molecular profiling for individual patients. In this way, they were able to observe divergent patterns in overall survival (OS) between males and females through different cancer entities in response to ICB. Regarding anti-PD-1/PD-L1 therapy male patients with colorectal cancer or glioblastoma multiforme showed increased survival, while female patients with esophagogastric cancer (ESCA) or NSCLC tended to have better OS. These gender differences were attributed to a number of factors including tumor mutation burden, neoantigen load, and mutation rates, which themselves showed gender disparity. More importantly, they demonstrated that it is of great importance to separate each cancer entity instead of pooling them together, as

this could be one of the biggest barriers in properly analyzing the influence of sex in ICB therapy response (158).

Altogether, the data concerning a sex-bias in the response to current ICI treatments are ambiguous at present. Reasons for this include multiple statistical caveats with meta-analyses including publication bias and inhomogeneity in the statistical design of clinical trials. Given the documented impact of sex on immune responses, it is hard to understand why in most ICI clinical trials a substantially larger fraction of males was included which could lead to bias in the results. Trials with equal numbers of males and females stratified for sex and/or separate trials in the different sexes are warranted especially in the context of anti-cancer immune therapies.

Altogether, the combination of ADT and immune therapy could open interesting therapeutic options especially in patients with androgen/AR-dependent cancers. Therefore, we now summarize the available clinical information about different immune therapies in prostate cancer with a focus on combination with ADT.

Combination of ICI and ADT in Prostate Cancer Patients

To date, many efforts have been made to integrate immunotherapy in the course of treatment of advanced prostate cancer. However, most immunotherapeutical approaches did not fulfill the high expectations. A rather "cold" tumor microenvironment and a low tumor mutation burden have been identified as potential causes. As mentioned above, ADT routinely used for advanced disease was found to influence the immune system in both, positive and negative ways (159).

So far, most trials have been carried out in androgen independent PCa characterized by disease progression despite testosterone values in the castration level due to ADT or orchiectomy. In fact, the only immunotherapy approved to date is Sipuleucel-T for asymptomatic or mildly symptomatic castration resistant prostate cancer (CRPC). Sipuleucel-T is an autologous cellular immunotherapy for which DCs are incubated *ex vivo* with a fusion protein consisting of prostate specific acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (160). In a phase 3 clinical trial (IMPACT; NCT00065442) with mCRPC patients, it was shown that in patients treated with Sipuleucel-T, the risk of death was significantly diminished and median overall survival (OS) was increased by 4.1 months vs. placebo-treated patients. However, due to company policy Sipuleucel T is only available in the USA and Canada.

In addition, different vaccination strategies were developed for prostate cancer. ProstVac VF (PRO; PSA-TRICOM), is a heterologous prime-boost regimen of two different recombinant pox-virus vectors that comprises a prime and multiple boosts with attenuated strains of vaccinia and fowlpox viruses, respectively. Both recombinant viruses encode human PSA and T-cell co-stimulatory proteins CD54, CD58, and CD80 (TRICOM). Remarkably, in a placebo-controlled phase 2 clinical trial of men with minimally symptomatic and chemotherapy-naive mCRPC, PROSTVAC was linked to a 44% decrease of death (161). In contrast, a double-blind, randomized phase 3 clinical trial evaluating 3 treatment groups (1) PRO+ Placebo, (2) PRO+GM-CSF, or (3) Placebo + Placebo showed no survival advantage with a median OS of 34.3, 33.3, and 34.2 months, respectively (162).

The first ICI examined in mCRPC was the CTLA-4 inhibitor ipilimumab (ipi). In a multicenter, randomized, placebo-controlled, double-blind, phase 3 trial ipi was evaluated in men with at least one bone metastasis originated from CRPC who had progressed after docetaxel therapy. Patients received first bone-directed radiotherapy and then were treated with either ipi 10 mg/kg or placebo every 3 weeks for up to four doses. Median overall survival was 11.2 and 10.0 months after ipi or placebo, respectively, with a trend for improval, but no significant advantage for patients treated with the immunotherapy (HR 0.85; $p = 0.053$) (163). In a second multicenter, double-blind, phase III trial, ipi was compared to placebo in chemotherapy-naïve mCRPC patients. Ipi 10 mg/kg or placebo were administered every 3 weeks for up to four doses followed by maintenance therapy in non-progressing patients every 3 months. Again, the study failed its primary endpoint with no survival advantage for Ipi (164).

Similarly, results of PD-1 inhibitors in unselected PCa patients have been rather disappointing. Thus, PD-1 inhibitor pembrolizumab (pembro) monotherapy in PD-L1 positive mCRPC achieved CR, PR and stable disease in only 2, 4, and 17% of the patients, respectively, while 58% of the men were primarily progressive (165). Results for PD-L1 negative patients were even worse with no CR, 3% PR and 63% progressive disease. Interestingly, an upregulation of PD-L1 was observed in patients developing resistance to AR targeting agent (ARTA) enzalutamide (enza) (166). Enza effectively inhibits androgen binding to its receptor, AR nuclear translocation and subsequent interaction with DNA. It is widely used for the treatment of advanced prostate cancer with approvals for the treatment of metastatic and non-metastatic CRPC as well as hormone sensitive PCa (Dez 2019; FDA only). As described above, androgen deprivation has been associated with T-cell tumor infiltration and activation as well as increased T-cell responses in preclinical models (93, 98). Consequently, the addition of pembro was evaluated in a phase 2 clinical trial in patients progressing on enza. Remarkably, a PSA-response >50% and radiological responses were observed in 18 and 25% of the patients [**Figure 1**; (167, 168)]. An expansion cohort with 30 additional men presented at last year's ESMO confirmed these results with PSA- and radiological responses in 20 and 22% of the patients, respectively (169). Based on these results, phase 3 clinical trials evaluating the combination of Pembro and Enza have been initiated in hormone sensitive and castration resistant advanced prostate cancer.

In addition, different combinational treatment strategies, e.g., with different immunotherapies or IO and chemotherapy are currently under investigation in mCRPC and showed first promising results (**Table 1**).

TABLE 1 | Ongoing clinical trials combining ADT and immunotherapies in prostate cancer.

Indication	Drug	Phase	Study	No. of patients; primary endpoint
mCRPC	Enzalutamide + Atezolizumab vs. Enzalutamide	III	NCT03016312, Imbassador 250	*n* = 771; OS
mCRPC	Enzalutamide + Pembrolizumab	II	NCT02312557	*n* = 58; PSA response
mCRPC	Enzalutamide + Pembrolizumab vs. Pembrolizumab	II	NCT02787005, Keynote 199	*n* = 370; ORR
mCRPC	Enzalutamide + PROSTVAC-F/V-TRICOM vs. Enzalutamide	II	NCT01867333	*n* = 57; TTP
mCRPC	Abiraterone Acetate + Prednisone + Ipilimumab	I/II	NCT01688492	*n* = 57; PFS, safety
mCRPC	Effect of fecal transplantation from responders to Pembrolizumab/Enzalutamide to non-responders	II	NCT04116775	*n* = 32; PSA response
mCRPC	4 arms: Pembrolizumab + Olaparib; + Docetaxel + Prednisone; + Enzalutamide; + Abiraterone + Prednisone	Ib/II	NCT02861573, Keynote 365	*n* = 400; PSA response, safety, ORR
mCRPC	Enzalutamide + Pembrolizumab vs. Enzalutamide + Placebo	III	NCT03834493, Keynote 641	*n* = 1,200; OS, PFS
mCRPC	Nivolumab + Bipolar Androgen Therapy (supraphysiological testosterone therapy)	II	NCT03554317, COMBAT-CRPC	*n* = 44; PSA response
mCRPC	3 arms: Nivolumab + Rucaparib; + Docetaxel + Prednisone; + Enzalutamide	II	NCT03338790, CheckMate 9KD	*n* = 330; ORR, PSA Response
mCRPC	Abiraterone + Prednisone + Apalutamide vs. Abiraterone + Prednisone + Apalutamide + Ipilimumab	II	NCT02703623	*n* = 198; OS, safety, AR response marker, PSA, CTCs
mCRPC	many arms, different solid tumors: AZD4635 + Durvalumab vs. Durvalumab	I	NCT02740985	295, incidence of DLT in solid tumors
mCRPC	Avelumab + Abiraterone or Enzalutamide	II	NCT03770455	*n* = 13; PSA response
mCRPC	Avelumab + Bempegaldesleukin + Enzalutamide	Ib/II	NCT04052204	*n* = 170; DLT, PSA response
mHSPC	Nivolumab + Degarelix vs. Nivolumab + Degarelix + BMS-986253	Ib/II	NCT03689699, MAGIC-8	*n* = 60; PSA response, safety
mHSPC	ADT + Docetaxel vs. ADT + Docetaxel + Nivolumab vs. ADT + Ipilimumab/Docetaxel + Nivolumab	II/III	NCT03879122, PROSTRATEGY	*n* = 135; OS
CSPC	Ipilimumab + GnRH Analog	II	NCT01377389	*n* = 30; progression after 6 months
CSPC	Enzalutamide + PROSTVAC-F/V-TRICOM vs. Enzalutamide	II	NCT01875250	*n* = 38; tumor growth
CSPC	Degarelix + Ipilimumab	II	NCT02020070	*n* = 16; PSA response
Oligometastatic PC	Abiraterone Acetate + Prednisone + leuprolide acetate + Pembrolizumab + SBRT+/– SD 1-01	II	NCT03007732	*n* = 42; PSA response
Oligometastatic PC, neoadjuvant	Degarelix + Pembrolizumab + cryosurgery	II	NCT02489357	*n* = 12; PSA response, safety
Localized PC, neoadjuvant	Degarelix + Cyclophosphamid + GVAX vs. Degarelix	I/II	NCT01696877	*n* = 29; CD8+ T-cell infiltration, adverse events
Localized PC, neoadjuvant	Enzalutamide + Pembrolizumab	II	NCT03753243	*n* = 32; PCR
Localized PC, neoadjuvant	Atezolizumab vs. Atezolizumab + Enzalutamide	II	NCT03821246	*n* = 51; change in $CD3^+$ T-cells

The table was adapted from Ozdemir and Dotto (6) and Taghizadeh et al. (170). The ClinicalTrials.gov database registry was searched for the terms "prostate cancer" and several CTLA-1 and PD-1, PD-L1 inhibitors. Not yet recruiting trials are not listed. PC: prostate cancer, mCRPC, metastatic castration resistant prostate cancer; mHSPC, metastatic hormone sensitive prostate cancer; CSPC, castration sensitive prostate cancer; ADT, androgen deprivation therapy; AR, androgen receptor; A2AR, adenosine A2A receptor; SBRT, stereotactic body irradiation therapy; OS, overall survival; PSA, prostate specific antigen; ORR, overall response rate; TTP, time to progression; DLT, dose limiting toxicities; PCR, pathological complete response.

CONCLUDING REMARKS

In summary, the androgen/AR axis plays a crucial role in both reproductive and non-reproductive tissues. AR signaling has been shown to directly and indirectly affect many immune cells types from innate and adaptive immunity. Overall, the effect of androgens is largely immunosuppressive, in terms of cell numbers and activation state. Furthermore, androgens/AR have been associated to poor prognosis in a plethora of cancer entities. However, deprivation of androgen signaling, has not

always led to convincing beneficial effects in patients except for prostate cancer, therefore, future studies are warranted to determine specific mechanisms taking place and identify better treatment strategies. Additionally, despite impressive advances in the field of cancer immuno-oncology, the effect of androgens in anticancer immunity is yet to be determined. More importantly, there is lack of knowledge regarding the effects of androgens in emerging therapies. Clinical studies should include the possible effects of sex in the trial design.

AUTHOR CONTRIBUTIONS

IB-B, MV-D, GA, MJ, and SL wrote the manuscript. IB-B and MV-D conceived and edited the figure. All the authors approved the submission of the manuscript.

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Androgen-Influenced Polarization of Activin A-Producing Macrophages Accompanies Post-pyelonephritic Renal Scarring

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Ascending bacterial pyelonephritis, a form of urinary tract infection (UTI) that can result in hospitalization, sepsis, and other complications, occurs in ∼250,000 US patients annually; uropathogenic *Escherichia coli* (UPEC) cause a large majority of these infections. Although UTIs are primarily a disease of women, acute pyelonephritis in males is associated with increased mortality and morbidity, including renal scarring, and end-stage renal disease. Preclinical models of UTI have only recently allowed investigation of sex and sex-hormone effects on pathogenesis. We previously demonstrated that renal scarring after experimental UPEC pyelonephritis is augmented by androgen exposure; testosterone exposure increases both the severity of pyelonephritis and the degree of renal scarring in both male and female mice. Activin A is an important driver of scarring in non-infectious renal injury, as well as a mediator of macrophage polarization. In this work, we investigated how androgen exposure influences immune cell recruitment to the UPEC-infected kidney and how cell-specific activin A production affects post-pyelonephritic scar formation. Compared with vehicle-treated females, androgenized mice exhibited reduced bacterial clearance from the kidney, despite robust myeloid cell recruitment that continued to increase as infection progressed. Infected kidneys from androgenized mice harbored more alternatively activated (M2) macrophages than vehicle-treated mice, reflecting an earlier shift from a pro-inflammatory (M1) phenotype. Androgen exposure also led to a sharp increase in activin A-producing myeloid cells in the infected kidney, as well as decreased levels of follistatin (which normally antagonizes activin action). As a result, infection in androgenized mice featured prolonged polarization of macrophages toward a pro-fibrotic M2a phenotype, accompanied by an increase in M2a-associated cytokines. These data indicate that androgen enhancement of UTI severity and resulting scar formation is related to augmented local activin A production and corresponding promotion of M2a macrophage polarization.

Keywords: urinary tract infection, activin A, follistatin, macrophage polarization, *Escherichia coli*

INTRODUCTION

Urinary tract infections (UTIs) are extremely common, affecting millions of people worldwide. Uropathogenic strains of *Escherichia coli* (UPEC) cause over 80% of UTIs, including both bladder infections (cystitis) and ascending infection of the kidneys (pyelonephritis). UTIs predominantly affect females, though infant and elderly males exhibit higher rates of UTI compared to similarly aged females (1–6). Males also exhibit higher morbidity and mortality than females in the setting of complicated UTI (4, 7). Upper-tract UTI in childhood carries risk for renal scarring, which in turn correlates with risk of chronic kidney disease, and end-stage renal disease later in life (8–14). Our prior studies in mice demonstrated enhanced UTI severity and scar formation in males compared with females, phenotypes shown to be dependent on androgen exposure (15, 16).

Macrophage recruitment, polarization, and function are important for the proper resolution of many bacterial infections. In a typical response, circulating monocytes are recruited to the site of infection upon signaling by damage-associated and pathogen-associated molecular patterns (DAMPs and PAMPs), and proinflammatory cytokines such as IL-6, IFNγ, and TNFα; these arriving monocytes initially differentiate, or polarize, toward proinflammatory (M1) macrophages (17–25). These M1 cells further secrete proinflammatory cytokines and chemokines, exert phagocytic activity, and induce neutrophil apoptosis (25–30). Reduction of local DAMP and PAMP quantities, along with an increase in neutrophil debris, and accumulation of T_H2 cytokines, including cytokines such as CXCL1, G-CSF, and IL-10 (27, 31–33), subsequently encourages these M1 macrophages to polarize toward alternatively activated M2 macrophages (34–38). M2a macrophages are activated by IL-4 and IL-13, and are considered pro-fibrotic (39–42). These cells secrete TGFβ1 and are involved in cell growth, repair, and matrix deposition. Immune complexes and IL-1β stimulate M2b polarization, which is involved in regulation of the immune and inflammatory responses (43, 44). M2c macrophages are stimulated by IL-10, are involved in phagocytosis and matrix remodeling, and typically signal resolution of the inflammatory response to an injury (45–49).

Activin A, a TGFβ superfamily member that is a homodimer of inhibin β_A, has been shown to be upregulated in several different systemic infection or injury models (50–56). In models of non-infectious renal injury, activin A signaling promotes renal scarring and fibrosis (55–59); in other systems, activin A has been shown to exert varying effects on macrophage polarization. For example, it encourages an M1 phenotype on unstimulated monocytes and macrophages *in vitro* (60–63) but pushes these cells toward a M2 polarization state when they are primed with LPS (64–68).

Testosterone signaling increases susceptibility to, and severity of, experimental pyelonephritis and renal scars in both male and female mice (69), while anti-androgen treatments are protective against UTI in mice and in women with polycystic ovary syndrome (16, 70, 71). Sex differences are also evident in the immune response to infection, and vary somewhat by model. Males tend to have more circulating M1 macrophages during infection (72), and dihydrotestosterone (DHT) can induce a prolonged M1 macrophage polarization state *in vitro* (73). Females typically exhibit more intense inflammatory responses to multiple microbial stimuli (including vaccines), and have more efficient phagocytic macrophages and increased levels of Toll-like receptors (TLRs) and pro-inflammatory cytokines (74, 75). In contrast, women taking oral contraceptives demonstrated a decrease in several pro-inflammatory cytokines (IFNγ, TNFα) after LPS stimulation (75), and testosterone stimulation has been shown to decrease the production of TLR4 in mice (76).

In mouse models of non-infectious renal injury, aberrant wound healing in males is characterized by increased leukocyte infiltrate and enhanced proteolysis of ECM, while castration promotes favorable wound healing (77, 78). Renal fibrosis in these models is also strongly associated with the presence of M2 macrophages (79–83); in fact, adoptive transfer of M2 macrophages after unilateral ureteral obstruction (UUO) promoted the accumulation of αSMA+ cells (indicative of fibrotic scarring), a phenotype that involved signaling by members of the TGFβ superfamily (84).

Here, we used C57BL/6 females treated with testosterone cypionate (TC) in order to investigate how activin A influences macrophage polarization during ascending pyelonephritis in the androgenized host. Although several studies have investigated how activin A affects macrophage polarization *in vitro* in the presence of LPS, data are sharply lacking on how these interactions transpire during *in vivo* infection. We determined that during ascending UPEC pyelonephritis, androgen exposure results in increased local activin A and promotes recruitment of activin A-producing leukocytes, particularly activin A+ monocytes and macrophages. Further, androgenized mice exhibited decreased local IFNγ and TNFα along with increased CXCL1 and G-CSF, associated with decreased local M1:M2 macrophage ratios throughout infection. In particular, androgen exposure caused a persistent increase in pro-fibrotic M2a macrophages during later stages of infection. This androgen-dependent skewing toward M2a macrophages promotes an environment of reduced bacterial clearance and enhanced renal scarring.

MATERIALS AND METHODS

Bacterial Strains

UTI89, a clinical cystitis isolate of uropathogenic *Escherichia coli* (UPEC) (85), was grown statically overnight in Luria-Bertani broth (LB; Becton Dickinson, Sparks, MD) at 37°C. Overnight cultures were centrifuged for 10 min at 7,500 × *g* at 4°C before resuspension in sterile phosphate-buffered saline (PBS) to a final density of ∼4×10^8 colony-forming units (CFU)/mL.

Animals

All animal protocols received prior approval from the Washington University Institutional Animal Care and Use Committee. Experiments were conducted in female C57BL/6 mice (#000664; Jackson Laboratories, Bar Harbor, ME) or, for immunofluorescence analysis, in female bigenic Gli1-tdTomato$^+$ mice, which harbor a tamoxifen-inducible Cre for tdTomato

production from the Gli1 promoter [kind gift of B. Humphreys; (86)]. For androgenization, mice of either strain were given weekly intramuscular injections of 150 mg/kg testosterone cypionate (TC, Depo-Testosterone; Pfizer, New York, NY) beginning at 5 wk of age, and continuing until sacrifice. UTI was initiated by inoculation of the bladder with 1–2 $\times$ 10^7 CFU of UPEC *via* catheter at 7 wk of age, as described previously (87, 88).

Determination of Bacterial Loads

At the indicated time points, mice were anesthetized with inhaled isoflurane (Patterson Veterinary, Greeley, CO) and terminally perfused with 4°C PBS through the left ventricle. Bladders and kidneys were aseptically removed and homogenized in 4°C PBS. The resulting tissue homogenates were serially diluted and plated on LB agar.

Tissue Preparation and Histology

Gli1-tdTomato$^+$ Mice were euthanized as described above, and aseptically removed kidneys were fixed in 4% paraformaldehyde in PBS for 1 h at 4°C, incubated overnight in 30% sucrose in PBS at 4°C, then embedded in OCT (Fisher Scientific, Hampton, NH). Embedded kidneys were cryosectioned into 5–8-μm sections and mounted onto Superfrost Plus slides (Fisher Scientific). For immunofluorescence staining, sections were washed with PBS, blocked with 10% fetal bovine serum (FBS) in PBS, then stained with fluorescently conjugated primary antibodies against CD206-Alexa Fluor 488 (1:200; Biolegend #141709) and CD80-APC (1:200; Biolegend #104713). Sections were then washed with PBS, stained with 1:5,000 4′,6-diamidino-2-phenylindole (DAPI) and mounted with ProLong Gold (both from Life Technologies, Carlsbad, CA). Images were captured digitally with a Zeiss LSM 880 Airyscan confocal microscope (Oberkochen, Germany).

Flow Cytometry

Kidneys were harvested as described above, and were manually homogenized into cold RPMI (Gibco) before treatment with RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$) at room temperature to ensure complete lysis of any remaining RBCs. After washing, cells were subjected to a Percoll gradient (Percoll PLUS; GE Healthcare, Uppsala, Sweden) in FACS buffer [10% FBS, 1% w/v sodium azide, 2 mM ethylenediaminetetraacetic acid (EDTA) in PBS] + 25 mM sucrose for leukocyte enrichment, then resuspended in 4°C PBS and stained with Live/Dead Fixable Yellow (ThermoFisher Scientific). Cells were washed again, resuspended in 4°C FACS buffer and blocked with Fc Block (BD Biosciences, San Jose, CA) on ice, followed by staining with fluorescently conjugated antibodies against the following extracellular antigens: CD45-BV510 (1:200; BD Biosciences #563891), NK1.1-AlexaFluor 700 (1:50; Biolegend #108730, San Diego, CA), CD11c-AlexaFluor 700 (1:200; Biolegend #117320), Ly6G-AlexaFluor 700 (1:200; Biolegend #127621), CD19-AlexaFluor 700 (1:200; Biolegend #115527), CD3e-AlexaFluor 700 (1:100; BD Biosciences #557984), CD150-APC (1:100; Biolegend #115910), CD206-PE-Cy7 (1:100; Biolegend #141719), CD86-PE-Cy5 (1:100; Biolegend #105016), CD115-PE (1:100; Biolegend #135506), CD80-FITC (1:50; Biolegend #104706). After staining, cells were washed, fixed in 4% paraformaldehyde in PBS, permeabilized on ice with Perm/Wash buffer (10% FBS, 1% w/v sodium azide, 1.3 mM saponin in PBS, pH 7.4–7.6), and then stained with the intracellular antibody Inhibin β_A-MaxLight405 (1:20; US Biological #211496, Salem, MA). All macrophages described are CD11b+ and Ly6C+. M1 macrophages are defined as CD80+, F4/80+, MHC-II lo. M2a macrophages are defined as CD206+ F4/80+ and MHC-II lo/−; M2b as CD86+, F4/80+/−, MHC-II lo/−; and M2c as CD150+, F4/80+/−, MHC-II hi (data not shown). For flow cytometry of whole-kidney activin A production, the kidneys were processed as described above, but cell suspensions were not subjected to the Percoll gradient. After blocking, cells were stained with labeled antibodies against the following extracellular antigens: E-cadherin (CD324)-PE-Cy7 (1:200, Biolegend #147309), and CD45 (30-F11)-BV510 (1:200, BD Biosciences #563891) and the intracellular antibody Inhibin β_A-MaxLight405 (1:20; US Biological #211496) as described above. Stained cells were washed, resuspended in FACS buffer and subjected to flow cytometry on a LSR II Fortessa instrument (BD Biosciences). Results were analyzed using FlowJo software (BD Biosciences). A representative gating scheme is provided in **Figure S1**.

Immunoblotting

Harvested kidneys were flash frozen in liquid nitrogen and stored at −80°C until use. Kidneys were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% v/v Nonidet P-40, 0.1% w/v SDS, 0.5% w/v sodium deoxycholate, pH 7.4) containing PhosSTOP phosphatase inhibitor (Roche; Basel, Switzerland) and complete Mini protease inhibitor (Roche). The lysates were cleared by centrifugation (2 $\times$ 5 min at max speed in a tabletop centrifuge), followed by total protein quantification by BCA assay (Invitrogen, Carlsbad, CA). Eighty μg of protein was run on SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% w/v non-fat milk (Carnation, Vaud, Switzerland) in PBS containing 0.05% v/v Tween-20 (PBST), and probed with primary antibodies against follistatin (1:500; Invitrogen # PA5-79284) and CoxIV (1:20,000; Cell Signaling Technologies #4844, Danvers, MA) in blocking buffer overnight at 4°C. Membranes were washed and probed 1:2,000 with the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare #NAP34) in blocking buffer for 1 h at room temperature. Membranes were washed again and developed with the Clarity Western ECL Kit (Bio-Rad, Hercules, CA).

Cytokine Quantification

Protein was extracted from flash-frozen kidneys as described above, and diluted in PBS to 900 μg/mL. The diluted protein was analyzed with a customized Bio-Plex Pro Mouse Cytokine Group I kit (Bio-Rad) according to the manufacturer's instructions. The plate was read with a Bio-Plex 200 system and analyzed using BioPlex Manager 6.1 software.

qPCR

mRNA was extracted from flash-frozen kidneys using RNA Stat-60 (amsbio, Cambridge, MA) according to package instructions. One μg mRNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to package instructions. qPCR was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), containing ∼20 ng of cDNA and 350 nM primers. Thermal cycling was performed on a 7500 Fast RT-PCR system (Applied Biosystems, Foster City, CA) with the following protocol: 95°C, 3 min; 40 × (95°C, 10 s; 60°C, 30 s). A list of primer sequences is provided in **Table S1**.

Statistical Analysis

Statistical analysis for CFU and Bio-Plex data was performed using the non-parametric Mann-Whitney U-test. All other statistics were performed with an unpaired *t*-test. $P < 0.05$ were considered significant.

RESULTS

Androgen Exposure Amplifies Renal Activin Expression During Pyelonephritis

In agreement with our previous work (16, 69), TC-treated (androgenized) mice maintained consistently high UPEC titers in both bladders and kidneys, significantly higher than those in vehicle-treated mice beginning 14 days post infection (dpi; **Figure 1**). As infection progressed, kidneys of TC-treated mice had increased global transcription of *Inhba* (encoding activin A) beginning 14 dpi and continuing through 28 dpi (**Figure 2A**). This increased transcription led to modest but statistically significant increases in activin A production 28 dpi by both epithelial (CD45– E-cadherin+; **Figure 2B**) and non-epithelial cells (CD45– E-cadherin–; **Figure 2C**), as determined by flow cytometry. This increase in activin A is consistent with similar increases seen in other renal injury models (55, 56). Meanwhile, the leukocyte (CD45+) population in TC-treated mice showed a significant elevation of activin A production 14 dpi (**Figure 2D**). This activin burst was of much greater amplitude than that seen in the other cell populations, leading us to investigate further how activin production by leukocyte populations could associate with the reduced UPEC clearance and enhanced scar formation seen in the androgenized host.

Follistatin Production Is Suppressed in Androgen-Exposed Mice With UTI

Follistatin binds strongly to activin A in the circulation and tissues, preventing its binding to its cellular receptor and thereby rendering it inactive (89–91). We hypothesized that renal tubular epithelial cell death associated with UPEC infection would reduce local production of follistatin (16). Indeed, while whole-kidney transcription of follistatin during UPEC infection was not altered in TC-treated mice (**Figure 3A**), follistatin production in whole-kidney homogenates was significantly reduced in TC-treated mice 10 and 14 dpi, as measured by quantitative immunoblot (**Figures 3B,C**). There was mild (but not statistically significant) reduction in follistatin production in androgenized mice across the other sampled time points (**Figure 3C**). Taken together, increased activin A production, coupled with decreased follistatin production, would provide an environment in the androgenized mouse kidney with increased activin A activity during UPEC infection.

Androgenized Mice Harbor Increased Activin A-Producing Myeloid Cells in the Infected Kidney

Activin A has been shown to affect macrophage polarization *in vitro*, encouraging M1 polarization in unstimulated macrophages while promoting M2 polarization in LPS-stimulated models (56–64). We examined leukocyte (CD45+) populations within the kidneys of TC-treated mice at various time points in order to interrogate the role of androgens in activin A-driven macrophage

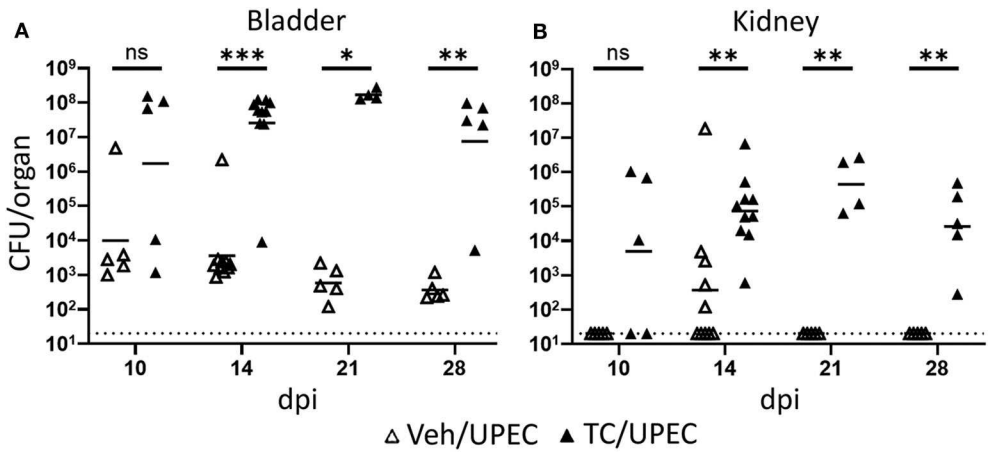

FIGURE 1 | Androgenized mice exhibit severe UTI. Organ titers (CFU) were quantified in serially diluted bladder **(A)** or kidney **(B)** homogenates at the indicated time points post UPEC infection of vehicle-treated mice (open triangles) or TC-treated mice (filled triangles). Dotted line indicates the limit of detection; dpi, days post-infection. $n = 4$–10 mice per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

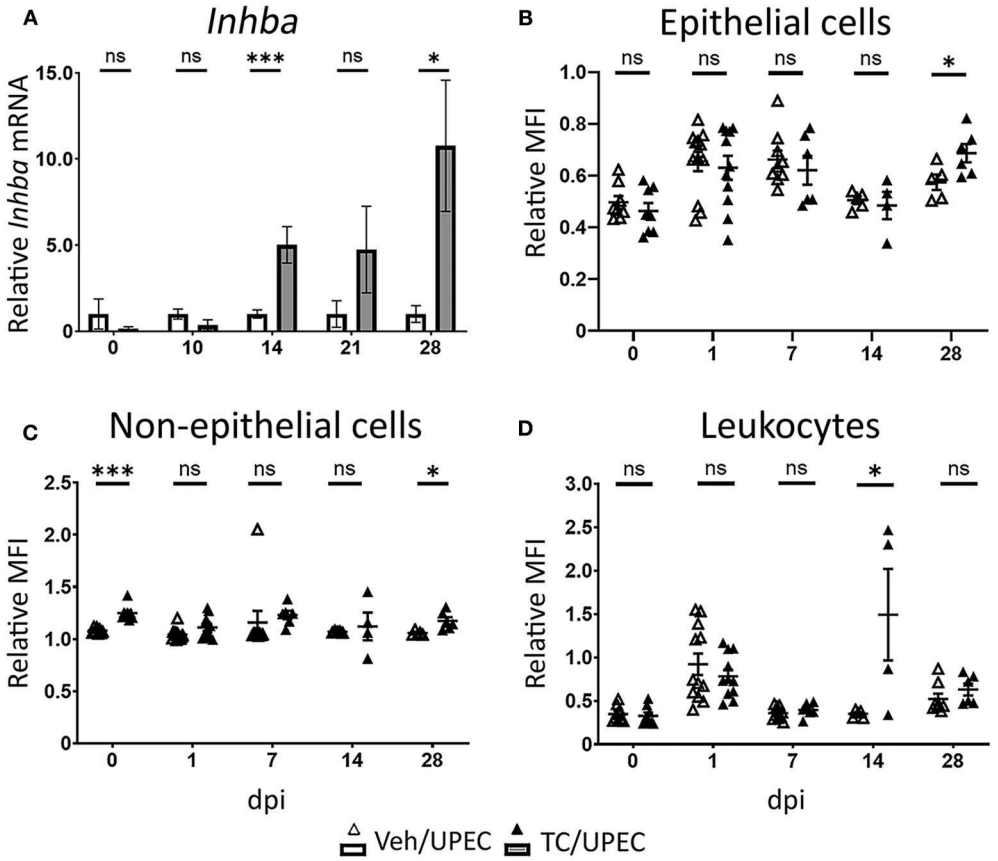

FIGURE 2 | Activin A expression and production is increased in the kidneys of androgenized mice. **(A)** Relative whole-kidney mRNA expression of *Inhba* was determined in vehicle-treated mice (open bars) and TC-treated mice (filled bars) by qPCR at various time points post UPEC infection. $n = 4$–8 mice per group. The relative mean fluorescence intensity (MFI) of activin A in **(B)** epithelial cells (CD45− E-cadherin+), **(C)** non-epithelial cells (CD45− E-cadherin−), or **(D)** leukocytes (CD45+ E-cadherin−) compared to the MFI in the total live cell population was determined by flow cytometry at the indicated time points in vehicle-treated mice (open triangles) or TC-treated mice (filled triangles). $n = 4$–10 mice per group. $^{*}P < 0.05$, $^{***}P < 0.001$.

polarization during pyelonephritis. After 14 dpi, TC-treated mice consistently exhibited increased recruitment of CD45+ cells to the kidneys compared to vehicle-treated mice (**Figure 4A**). While most of these CD45+ cells were neutrophils (Ly6G+; data not shown), TC-treated mice displayed a sustained increase in both monocyte (CD19− CD3e− Ly6G− CD11c− NK1.1− CD115+) and macrophage (CD19− CD3e− Ly6G− CD11c− NK1.1− CD115−) populations in the kidneys starting 14 dpi (**Figures 4C,E**). There were also more activin A+ leukocytes, monocytes, and macrophages in the kidneys of androgenized mice, indicating that both the monocyte and macrophage populations were contributing to activin A signaling in the infected kidney (**Figures 4B,D,F**).

Androgen Exposure Favors Polarization of Renal Macrophages Toward the Pro-fibrotic M2a Phenotype

To investigate how the increased levels of activin A affected macrophage polarization during UPEC infection and resolution, we quantified kidney macrophages in the M1 or M2 polarization states at various time points. Compared with vehicle-treated mice, androgenized mice harbored an increased population of M1 macrophages (CD80+; **Figure 5A**) in the kidneys 14 and 21 dpi, and an even greater increase in M2 macrophages from 14 to 28 dpi (CD80−; **Figure 5B**). This led to an overall decrease in the M1:M2 ratio, beginning 10 dpi and sustained throughout the course of infection (**Figure 5C**). A prolonged reduction in the M1:M2 ratio is reflective of aberrant wound healing and is associated with fibrotic scarring (25).

Within the population of activated macrophages, the M1 phenotype predominated in both vehicle and TC-treated mice throughout the course of infection; however, androgenized mice showed a significant reduction at multiple time points in the fraction of polarized macrophages that were M1 (**Figure 6A**). Correspondingly, androgenized mice exhibited a significant increase in M2a (CD206+, CD150−) macrophages, beginning 14 dpi and persisting through the remainder of the course (**Figure 6B**). Both M1 and M2a macrophages were visualized near populations of Gli1+ activated myofibroblasts, which are the major producers of extracellular matrix proteins in fibrotic injury (**Figure S2**) (86, 92). Vehicle- and TC-treated mice showed

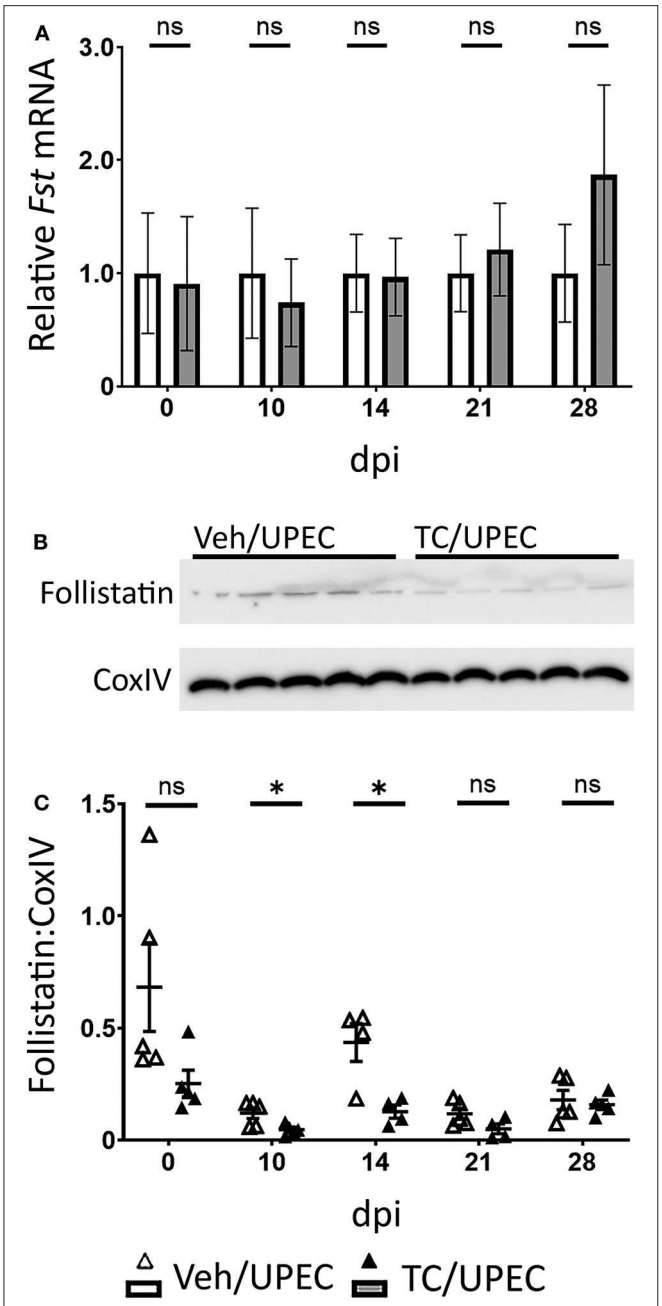

FIGURE 3 | Follistatin production is reduced during pyelonephritis in androgenized mice. **(A)** Relative whole-kidney *Fst* mRNA was measured by qPCR at the indicated time points in vehicle-treated mice (open bars) and TC-treated mice (filled bars). $n = 4$–8 mice per group. Whole-kidney protein production of follistatin was determined by quantitative western blot [representative blot shown in **(B)**; quantitation in **(C)**] at the indicated time points in vehicle-treated mice (open triangles) and TC-treated mice (filled triangles). $n = 4$–5 mice per group. $^*P < 0.05$.

equivalent increases in M2b (CD86+) macrophages at later time points following infection (**Figure 6C**). Vehicle- and TC-treated mice also harbored similar proportions of M2c (CD150+) macrophages in the kidneys until 28 dpi, when androgenized mice had significantly more (**Figure 6D**). These results indicate that androgens promote activin A production by myeloid cells responding to UPEC pyelonephritis, with a corresponding increase in M2a polarization of renal macrophages.

Androgens Promote M2a-Associated Cytokine Expression During Pyelonephritis

M2a macrophages have been associated with tissue fibrosis after non-infectious injury (39, 40, 93, 94). These cells secrete a number of cytokines and chemokines involved in immunomodulation and repair, including TGFβ1, a chief signaling factor in renal fibrosis (84, 95, 96). Further, adoptive transfer of M2a macrophages led to reduced healing and increased fibrosis of endometriotic lesions (97). We investigated cytokine content in the kidneys of vehicle and TC-treated mice throughout infection. Notably, among M1-associated cytokines, IFNγ was significantly reduced in androgenized mouse kidneys 10 dpi (**Figure 7A**), while TNFα was unaltered by androgen exposure (**Figure 7B**). Meanwhile, M2-activating cytokines CXCL1 and G-CSF were significantly increased in TC-treated mice at multiple time points (compared with vehicle-treated; **Figures 7C,D**), indicating that the cytokine profile of the infected, androgenized kidney may help to drive recruited macrophages toward the M2 polarization state. In line with the flow cytometry data (**Figure 6B**), TC treatment did not alter the level of M2b stimulant IL-1β in the kidneys (**Figure 7E**) and acted to depress production of the M2c stimulant IL-10 (**Figure 7F**). This lack of increase in IL-1β and IL-10 may discourage progression of M2a macrophages toward the M2b and M2c phenotypes that would characterize an optimal healing process.

DISCUSSION

Our published studies showed that testosterone exposure favors the development of severe pyelonephritis in both C3H and C57BL/6 mice (16, 69), with exacerbation of post-pyelonephritic scarring. The present work demonstrates that androgens encourage a reduction in pro-inflammatory M1 macrophages in the UPEC-infected kidney, conversely favoring the sustained presence of pro-fibrotic M2a macrophages, prolonging UTI and offering a cellular basis for the altered resolution and enhanced scarring we demonstrated previously.

Activin A, a member of the TGFβ superfamily, is involved in both healing and renal fibrosis in several models (55–59) and is a major driver of macrophage polarization (56–64). TC-treated mice demonstrated an increase in *Inhba* transcription and activin A production throughout their kidneys, with a corresponding decrease in follistatin. The cumulative result of these effects is more active activin A in the kidneys of androgen-exposed mice. Interestingly, the CD45+ leukocyte population in TC-treated mice showed the most pronounced increase in activin A (14 dpi); correspondingly, infiltration of multiple myeloid lineages was enhanced in androgenized mice, and the number of activin A-producing cells in these groups also steadily increased.

Activin A signaling has been shown to encourage recruited monocytes to differentiate into either pro-inflammatory M1

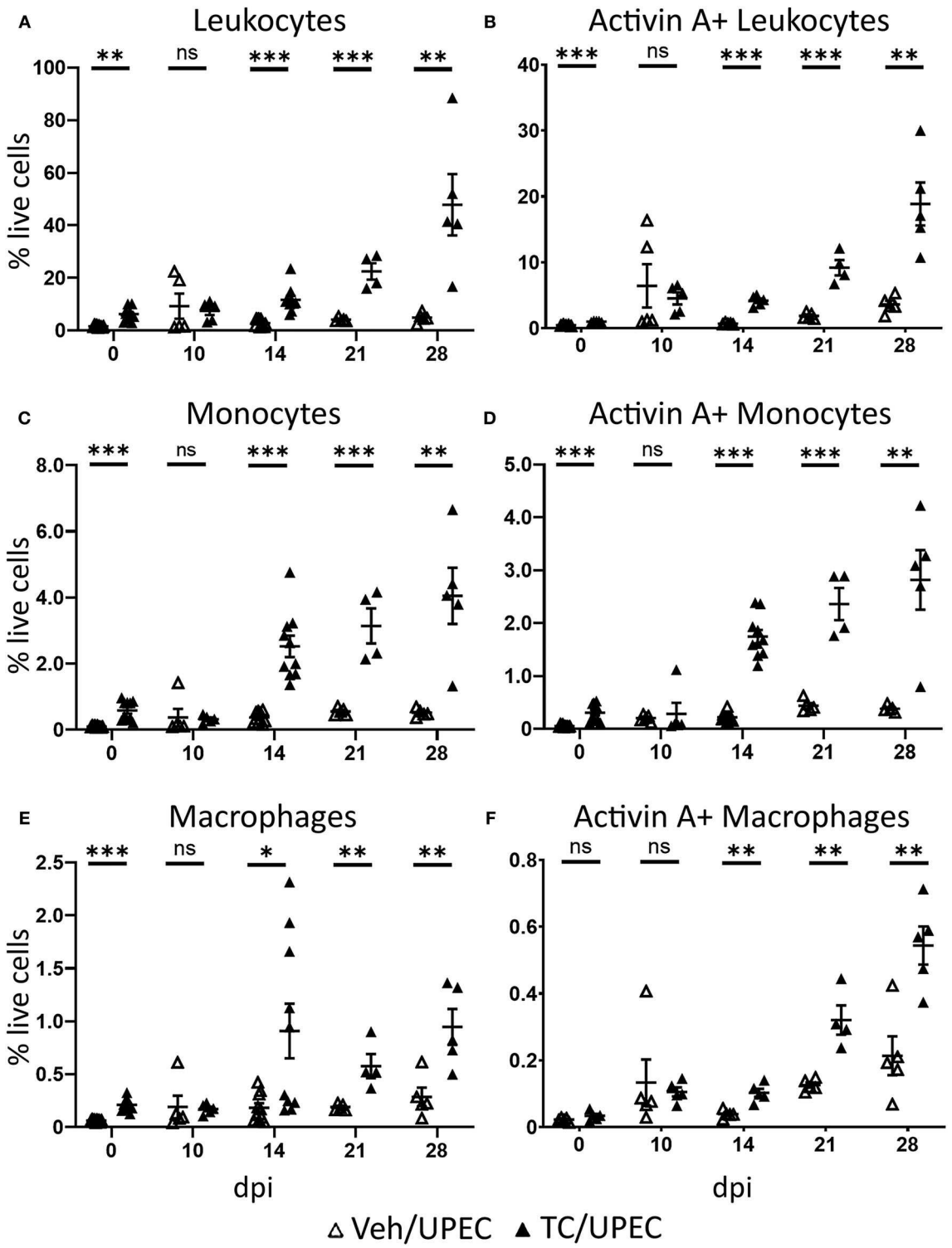

FIGURE 4 | Androgenized mice have larger populations of activin A-producing leukocytes, including monocytes and macrophages, in the kidneys during UPEC infection. The population of **(A)** total leukocytes (CD45+), **(B)** activin A+ leukocytes, **(C)** monocytes (CD45+ CD115+ CD19− CD3e− Ly6G− CD11c− NK1.1−), **(D)** activin A+ monocytes, **(E)** macrophages (CD45+ CD115− CD19− CD3e− Ly6G− CD11c− NK1.1−), and **(F)** activin A+ macrophages as a percentage of the total live cell population was determined by flow cytometry in vehicle-treated mice (open triangles) and TC-treated mice (filled triangles) at the indicated time points. $n =$ 4–10 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

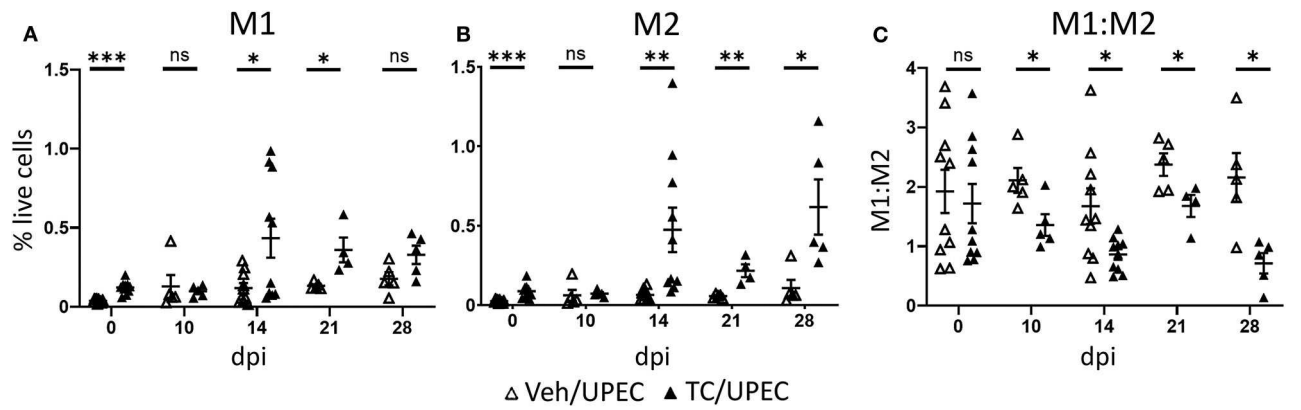

FIGURE 5 | Androgenized mice have increased populations of both M1 and M2 macrophages, but a reduced M1:M2 ratio. The populations of **(A)** M1 macrophages (CD80+) and **(B)** M2 macrophages (CD80−) as a percentage of total live cells was determined by flow cytometry at the indicated time points in vehicle-treated mice (open triangles) and TC-treated mice (filled triangles). **(C)** The ratio of M1 to M2 macrophages for each mouse was calculated from the data represented in **(A,B)**. $n =$ 4–10 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

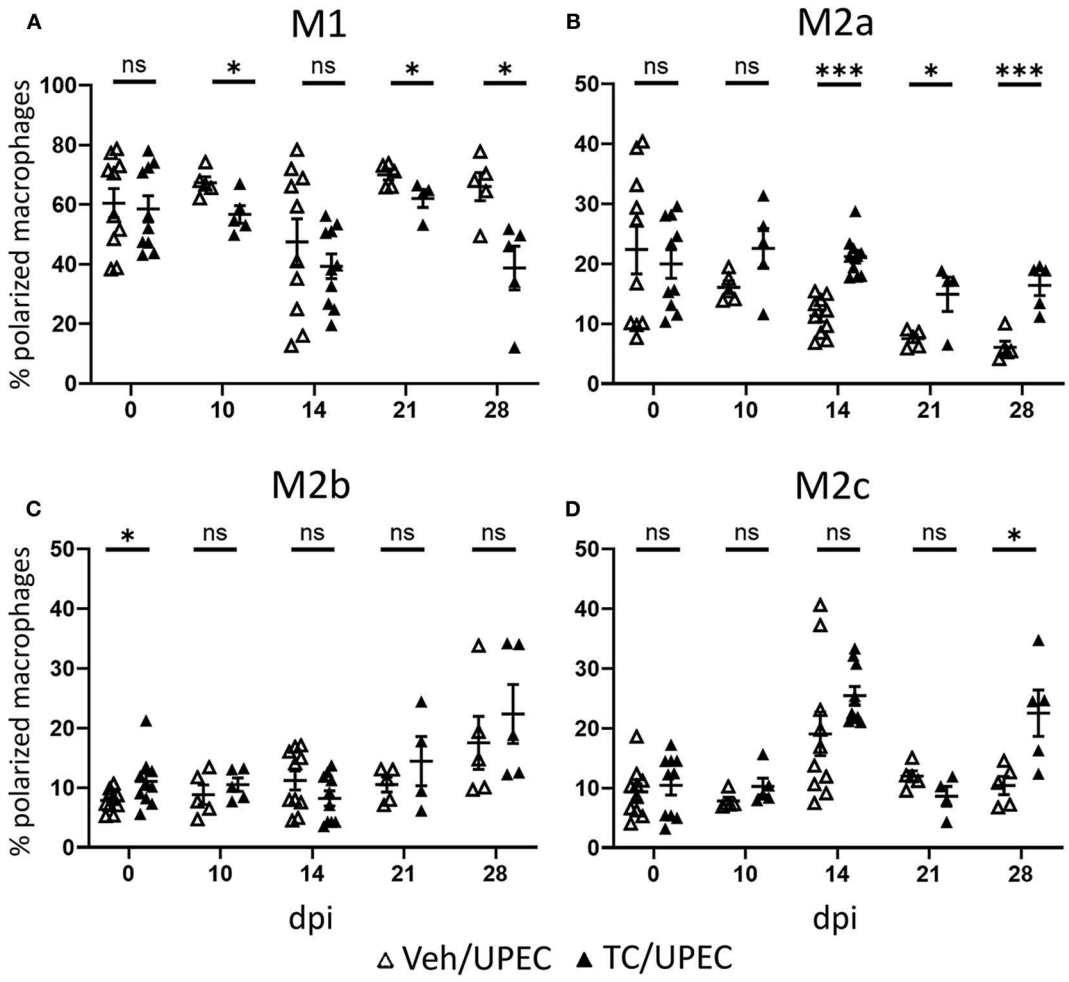

FIGURE 6 | Androgenized mice harbor an increased proportion of M2a polarized macrophages. The population of **(A)** M1 macrophages (CD80+), **(B)** M2a macrophages (CD80− CD206+ CD150−), **(C)** M2b macrophages (CD80− CD86+), and **(D)** M2c macrophages (CD80− CD150+) as a percentage of the polarized macrophage population was determined by flow cytometry at various time points in vehicle-treated mice (open triangles) and TC-treated mice (filled triangles). $n =$ 4–10 mice per group. $^{*}P < 0.05$, $^{***}P < 0.001$.

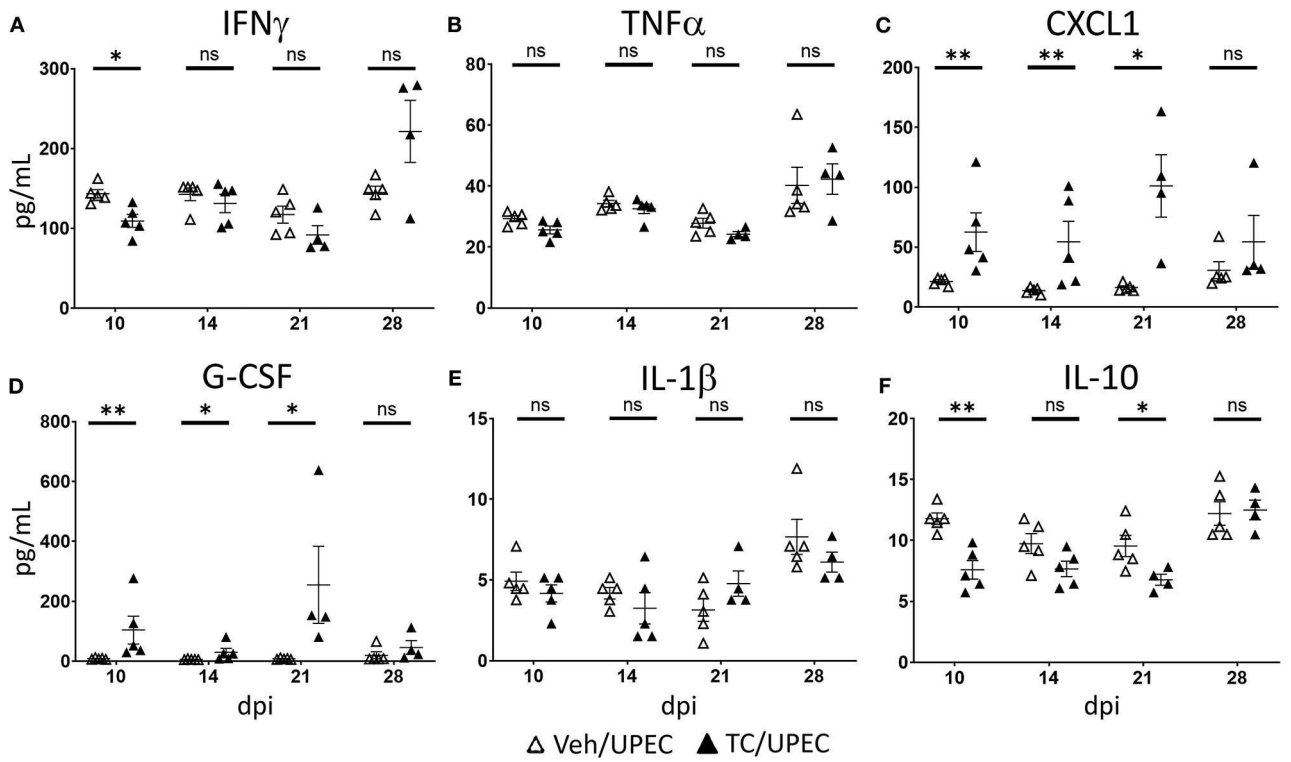

FIGURE 7 | Kidneys of androgenized mice contain reduced M1- and increased M2-polarizing cytokines. The concentrations of **(A)** IFNγ, **(B)** TNFα, **(C)** CXCL1, **(D)** G-CSF, **(E)** IL-1β, and **(F)** IL-10 were quantified by Bio-Plex assay from protein extracted from whole kidneys of vehicle-treated mice (open triangles) and TC-treated mice (filled triangles) at the indicated time points post-infection. $n = 4$–10 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$.

macrophages or alternatively activated M2 macrophages (98). This variance in polarization states appears to be environmentally dependent, with unstimulated monocytes and macrophages favoring an M1 phenotype (56–59), while LPS stimulation before activin A treatment skews these cells toward an M2 phenotype (60–64). During active bacterial infection, as in our model, the kidney is exposed to extensive LPS stimulation. This, combined with the increase in activin A, caused androgenized mice to have a sustained preponderance of M2 macrophages. When we examined the specific polarization states of these M2 cells, we found that TC-treated mice harbored significantly more M2a macrophages at all time points beginning 14 dpi. Macrophage polarization and proliferation occurs within the injured kidney, and M2 macrophages are highly important for repair of non-infectious renal injury (99–101). Specifically, M2a macrophages are known to be pro-fibrotic, enhancing TGFβ1 expression, cell growth, tissue repair, and matrix remodeling (39–42). During optimal recovery from tissue injury, this M2a population subsides as they differentiate toward (and are replaced by) immunoregulatory M2b and M2c macrophages, allowing the inflammatory response to abate and the affected tissue to return to a healed state (36, 96, 102, 103). In our model, while M2b and M2c numbers increased slightly over time in both TC- and vehicle-treated mice, the augmented M2a population in androgenized mice did not subside. The persistence of these M2a macrophages would act to prolong the pro-fibrotic state, prevent resolution of inflammation, and favor the androgen-enhanced renal scarring we have shown previously (15, 16).

Macrophage polarization is also highly dependent on secreted cytokines that are secreted by the injured tissue and the macrophages themselves (27, 96). M1 polarization occurs via stimulation with several pro-inflammatory signals (e.g., LPS and IFNγ, with ensuing TNFα, and IL-6 production) (15–23), as are normally elicited early after bacterial infection of the urinary tract (104–106). M2 macrophages are sensitive to a variety of Th2 cytokines, including CXCL1, G-CSF and IL-10 (27, 31–33). The whole-kidney cytokine profiles following UPEC infection aligned with the macrophage polarization states we observed, with androgenized mice exhibiting suppressed IFNγ and unaltered TNFα, accompanied by increased CXCL1 and G-CSF. The depressed IL-10 levels during infection in androgenized mice may hinder the adoption of M2b or M2c phenotypes, restraining kidney macrophages in a prolonged M2a state.

In total, our data indicate that testosterone exposure alters the typical response to renal UPEC infection, pushing the kidney toward a dysfunctional healing process through increased activin A signaling and altered cytokine release. These signals encourage the recruited monocytes to polarize toward and persist as M2a macrophages for weeks in the kidney, preventing bacterial clearance and proper resolution of inflammation. A deeper understanding of how testosterone regulates these signals may allow us to modulate this immune

response to help mitigate adverse long-term sequelae of severe pyelonephritis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee, Washington University School of Medicine.

AUTHOR CONTRIBUTIONS

TH, KH, and DH conceived the study. TH, CC, AD, and JG designed and performed experiments. DH and KH critically reviewed the data. TH generated the first manuscript draft. TH, DH, JG, and KH edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01641/full#supplementary-material

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The Effects of Androgens on T Cells: Clues to Female Predominance in Autoimmune Liver Diseases?

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The immune system responds differently in women and in men. Generally speaking, adult females show stronger innate and adaptive immune responses than males. This results in lower risk of developing most of the infectious diseases and a better ability to clear viral infection in women (1–5). On the other hand, women are at increased risk of developing autoimmune diseases (AID) such as rheumatoid arthritis, multiple sclerosis (MS), systemic lupus erythematosus (SLE), Sjögren's syndrome, and the autoimmune liver diseases autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC) (6). Factors contributing to the female sex bias in autoimmune diseases include environmental exposure, e.g., microbiome, behavior, and genetics including X chromosomal inactivation of genes. Several lines of evidence and clinical observations clearly indicate that sex hormones contribute significantly to disease pathogenesis, and the role of estrogen in autoimmune diseases has been extensively studied. In many of these diseases, including the autoimmune liver diseases, T cells are thought to play an important pathogenetic role. We will use this mini-review to focus on the effects of androgens on T cells and how the two major androgens, testosterone and dihydrotestosterone, potentially contribute to the pathogenesis of autoimmune liver diseases (AILD).

Keywords: testosterone, androgen, immunity, autoimmunity, androgen receptor, sex-bias, T cell, sex hormones

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ANDROGENS IN STEADY STATE

The androgenic steroid hormones, testosterone, dihydrotestosterone (DHT), androstenedione, and dehydroepiandrostenone (DHEA) are generated from cholesterol (7). In men, the majority of testosterone precursors (>95%) are produced by Leydig cells in the testes and, to a lesser degree, by the adrenal glands. In women, testosterone precursors are produced by the adrenal glands, the thecal cells of the ovaries, and, during pregnancy, by the placenta (7–10). Metabolism of androgens is complex with testosterone generated from androstenedione in peripheral tissues and the conversion of testosterone into estrogen mediated by the enzyme aromatase in a context and tissue specific manner. Conversion of testosterone into DHT mainly occurs in the liver by the action of 5α-reductase, and DHT cannot be further metabolized to estrogen (11). Sixty-five to 70% of testosterone in blood is bound to sex hormone-binding globulin (SHGB) and 30–35% to albumin, which transport the hormone to target tissues. Only around 0.5–3% of testosterone is found freely in blood (9). Concentrations of bioavailable testosterone can be estimated with total testosterone, SHGB, and albumin serum levels (12).

Interestingly, women show blood androgen levels that are higher than the levels of estrogen. This is due to DHEA produced by the adrenal glands which is subsequently converted to testosterone via androstenedione (8). The levels of total testosterone in women range from 0.35 to 2.94 nmol/l, and there are no significant changes during daytime in testosterone and free testosterone levels (9, 13). In premenopausal women testosterone and free testosterone slightly peak midcycle, but DHT levels do not seem to change during the menstrual cycle (14, 15). With age and after menopause, testosterone levels in women decline, leading to significantly lower levels of testosterone, free testosterone, DHT, and SHGB (13).

In men, testosterone helps to regulate a variety of physiological processes including muscle mass and strength, bone mass, fat distribution, libido, and the production of sperm, red blood cells, and immune cells (11). Due to the complex metabolism of androgens and their tissue and context dependent conversion into estrogen, it is difficult to delineate the action of specific androgens within a given tissue in humans *in vivo*. For example, studies suggest that the effect of testosterone on male bone mass occurs mainly through its conversion to estrogen (16, 17). Serum testosterone levels are significantly higher in men than in women and typically range from 6.2 to 32.1 nmol/l (18). During daytime, a slight decrease in testosterone levels toward the afternoon can be observed (18). Testosterone production in men typically decreases with age to approximately the lower end of the mean levels observed in middle-aged adult men (12, 18–20).

ANDROGENS SIGNAL THROUGH CYTOSOLIC ANDROGEN RECEPTOR (AR) AND NON-CLASSICAL MEMBRANE BOUND RECEPTORS (mAR)

Cytosolic Androgen Receptor (AR)

Androgens, including testosterone and DHT, reach their target cells and signal through androgen receptors. In addition to the classical cytoplasmic androgen receptor (AR), androgens can also bind and activate membrane androgen receptors (mAR) (21). DHT binds the AR with a higher affinity and lower dissociation rate than testosterone, while testosterone probably has a higher affinity to the mAR (11). The expression of androgen receptors has been reported in many different tissues, in epithelial and endothelial cells, and in a variety of innate and adaptive immune cells, including human and mouse T cells (22–24).

Abbreviations: AID, autoimmune diseases; SHGB, sex hormone-binding globulin; DHT, dihydrotestosterone; AR, androgen receptor; mAR, membrane bound androgen receptors; LBD, ligand-binding domain; DBD, DNA-binding domain; NTD, N-terminal domain; ARE, androgen response elements; PI3K, phosphoinositide-3-kinase; CREB, cAMP response element-binding protein; Treg, regulatory T cell; BPH, benign prostatic hyperplasia; ADT, androgen deprivation therapy; AIRE, autoimmune regulator; VAT, visceral adipose tissue; MS, Multiple sclerosis; PBC, primary biliary cholangitis; AIH, autoimmune hepatitis; SLE, systemic lupus erythematosus; EAE, autoimmune encephalomyelitis; AMAs, anti-mitochondrial antibodies; ANAs, antinuclear antibodies; EAO, experimental autoimmune orchitis.

The classical cytoplasmic AR is a member of the nuclear receptor superfamily and can act as a ligand-dependent transcription factor (25, 26). The human AR gene consists of 8 exons and is located on the X chromosome (27). It has a ligand-binding domain (LBD), a DNA-binding domain (DBD), and an N-terminal domain (NTD) (27). In an unbound state, the AR is residing in the cytoplasm in a complex with chaperons, heat-shock proteins, and cytoskeletal proteins (27, 28). The binding of ligands leads to a conformational change, receptor dimerization, and translocation to the nucleus (29). The NTD affects the transcriptional activity and the DBD permits the binding and recognition of androgen response elements (ARE) on target genes (27). The complex finally disassociates and returns to the cytoplasm (27). AR can also be post-translationally modified through phosphorylation, methylation, or ubiquitination, allowing for ligand-independent modulation of signaling (27, 29, 30).

Next to the regulation of gene transcription, AR interacts with PI3K (phosphoinositide-3-kinase), Src family kinase, and RAS GTPase (27). This interaction affects MAPK/ERK signaling and ERK translocates into the nucleus to affect transcriptional factors leading to adjustment of gene expression involved, e.g., in cell proliferation and survival (27, 28). In a complex but not yet fully elucidated process, mTOR, FOXO1, FOXO3a, HDAC3, STAT3, EGFR, and AKT were shown to be involved in non-genomic AR signaling (27, 28, 31–35).

Membrane Bound Androgen Receptors (mAR)

The zinc transporter ZIP9 (SLC39A9) has been identified as a membrane bound androgen receptor (mAR), interacting with several kinase pathways such as ERK1/2 and others (36–39). In human prostate cancer cells with overexpressed ZIP9 (PC-3-ZIP9) and breast cancer cells (MDA-MB-468), stimulation with testosterone leads to G proteins being activated, second messenger pathways, and elevation of intracellular free zinc, resulting in initiation of apoptosis and upregulation of pro-apoptotic genes such as BAX, p53, and Caspase-3 (36, 40). In the spermatogenic cell line GC-2, testosterone was shown to induce activation of ERK1/2 and the transcription factors ATF-1 and CREB through Zip9, which interacted with G-protein Gnα11 (38, 39).

The G-protein coupled receptor GPRC6A was suggested as another mAR, which has not yet been reported in a broad range of tissues. *In vitro*, GPRC6A phosphorylates ERK after testosterone stimulation in prostate cancer and bone marrow stromal cells (11, 41). One group showed the involvement of GPRC6A in testosterone production in Leydig cells (42). To our knowledge, however, the expression of GPRC6A in T cells is unknown, reflecting the general lack of knowledge on the role of membrane bound androgen receptors in the immune system.

Androgen Independent Receptor Signaling

AR signaling can also be induced independently from androgen binding. In prostate cancer cells, IL-6 dependent interplay with AR interferes with the PKA/PKC/MAPK pathway and IL-8 has been shown to promote their AR dependent

growth and activation independent of androgens (11, 28, 42–44). Furthermore, IGF-1 stimulated AR phosphorylation, translocation to the nucleus, and upregulation of AR gene expression in myoblast C2C12 cell line (45, 46). These data suggest that inflammation associated changes in the cytokine milieu in an organ affected by autoimmune injury may significantly alter AR signaling. The liver is the central organ of androgen conversion, but so far, the effects of liver inflammation on testosterone metabolism and AR signaling have not yet been explored.

Moreover, the length of the CAG repeat region in exon 1 of the AR gene influences its signaling activity (47–49). Studies in men and women with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) demonstrated variable and sex dependent effects of this heritable trait on disease severity and phenotype (50–53).

Overall, the activation and signaling of AR and mAR is complex, and crosstalk between AR transcriptional activity and non-genomic modification of AR- or mAR induced signaling cascades can lead to highly context dependent modification of androgen responses (27).

ANDROGENS AND HUMAN T CELLS

AR expression was identified in the majority of innate and adaptive immune cells suggesting that androgens directly modulate the function and development of immune cells. Already in the 1980s, AR expression was reported for human thymocytes (54). Thereafter, AR was found to be expressed on various human and mouse cells of the innate immune system, such as monocytes and macrophages from different tissues, ILC2 progenitors, neutrophils, and mast cells (55–61). In adaptive immunity, AR-expression was shown in human T cells, including CD8+ T cells and CD4+ and splenic CD4+ CD25+ T cells (55, 56, 62–64). In addition to AR, CD4+ and CD8+ T cells were shown to express mAR (65).

The effects of androgens on T cells were studied *in vitro* and by comparing male and female T cells *ex vivo*. It was found that Foxp3 expression, the Treg master transcription factor, was increased in human T cells after DHT treatment *in vitro*, and increased Treg frequencies were reported in men compared to women, and in boys already at the age of eight (66–68). Therefore, androgens may already influence the frequencies of T cells *in vivo* early in life. In adult men, there is a recent report of a negative correlation between CD3+, CD8+, and CD4+ T cells residing in adipose tissue and serum testosterone levels (69). Moreover, upon stimulation of healthy human PBMC with TLR8/9 ligands, secretion of IL-10 in male PBMC was higher than in female PBMC. Upon TLR7 stimulation, IFNα was lower in male PBMC. The amount of IL-10 upon TLR9 stimulation correlated to dehydroepiandrosterone sulfate levels in males, but this study cannot conclude whether these are direct or indirect effects on T cells via dendritic cells (70). Microarray analysis of restimulated T cells showed a higher expression of "pro-inflammatory" genes, such as IFNγ, IL12Rß2, LTß, GNLY, and GZMA in female T cells, while male T cells had a higher expression of IL10, IL5, and IL17A (71). Moreover, healthy male human naïve CD4 cells produced lower levels of IFNγ and had a trend of higher levels of IL-17A upon CD3/CD28 stimulation, possibly through upregulated PPARα and downregulated PPARγ1, and similar results were observed in mice (72–74).

Analysis of men under hormone replacement therapy could give new insight into the effects of androgens *in vivo,* although it is impossible to delineate these *in vivo* effects to single immune cell types such as T cells. Thus, in hypogonadal men a reduction in serum IL1ß and TNF, as well as an increase in IL-10, has been described following testosterone replacement treatment. Whether part of these observed differences related to changes in T cell subpopulations remained speculation (75). In a single case study with one hypogonadal man, an increase in naïve CD4+ CD45Ra+ cells could be observed that could be reverted upon androgen treatment (76). In prostate tissue of BPH (benign prostatic hyperplasia) patients undergoing 5α-reductase type II inhibitor treatment with finasteride leading to reduced intraprostatic DHT levels, a stronger infiltration of CD8+ T cells and higher CCL5 expression was observed (77). Moreover, in a follow-up study, the authors showed *in vitro* that in conditions of low androgen concentrations, CD8+ T cells were able to promote prostate epithelial cell proliferation, possibly through the CCL5/JAK-STAT5/CCND1 pathway (78). After androgen deprivation therapy (ADT) of prostate cancer patients, Wang et al. found enrichment of CD4low HLA-G+ T cells in peripheral blood, besides generally increased CD4+ T cell frequencies (79). In detail, these CD4low HLA-G+ T cells expressed IL-4, IL-17A, and RORγt, indicating an enrichment of IL-4 producing TH17 cells after ADT (79).

Testosterone therapy in transgender individuals offers further possibilities to study the effects of androgens on immune cells *in vivo*. Giltay et al. reported an increase in the IFNγ/IL-4 ratio and TNF production of PBMCs isolated from women undergoing hormone replacement therapy with testosterone. Cells were stimulated with PHA for 36 h and the results indicated increased TH1 differentiation (80). However, as these results contrast some of the above-mentioned studies, they should be further validated and it should be investigated in detail which cell type produced these cytokines.

Taken together, these results provide evidence that androgens influence T cell function and phenotype either directly or indirectly. However, in-depth and comprehensive analyses of direct and context dependent androgen effects on human T cells are lacking.

EFFECTS OF TESTOSTERONE ON T CELLS IN ANIMAL MODELS

Animal models have added to the knowledge on the effects of testosterone on immune cells. Olsen et al. observed a reduced thymus size within 2–4 h after testosterone injection of castrated male mice already in 1998. Mechanistically, increased apoptosis was induced in *in vitro* thymus tissue culture through the AR and reduced percentages of CD4+ CD8+ double positive thymocyte were detected in testosterone treated mice (81, 82). However, several other studies found no direct *in vitro* effect of testosterone on apoptosis of isolated thymocytes (82, 83).

A potential explanation for this discrepancy could be that the thymic effects of androgens are mediated by AR expression on thymic epithelial cells (TEC) which are crucial for the negative selection of immature T cells (22, 84, 85). Reduction of androgen levels through castration of mice led to increased numbers of immature triple negative T cells and early T lineage progenitors and a decrease in mature CD4+ and CD8+ single positive cells in the thymus (86). More recently, thymic expression of AIRE (autoimmune regulator) in medullary TECs, which is involved in the thymic selection of T cells by clonal deletion of autoreactive T cells, has been reported to be higher in male human and mouse thymus, possibly induced by the effects of androgens through AR (87). However, ADT by castration of adult male mice did not change TCR diversity but increased the numbers of "naïve" CD44low CD4+ and CD8+ T cells within lymph nodes (88). Additionally, these mice recovered their T and B cells quicker than non-castrated controls after chemotherapy-induced lymphocyte depletion, and these androgen-deprived T cells were more prone to proliferate *in vitro* (88). Taken together, these data suggest that androgens affect T cell maturation and selection within the thymus either directly or indirectly via epithelial cells.

Regarding peripheral mature T cells, it has been shown that DHT treatment in female mice resulted in decreased IL-12 and increased IL-10 production compared to cells from untreated mice following aCD3 stimulation *in vitro*, and this difference was primarily caused by CD4+ T cells (55). Microarray analysis of splenic CD4+ T cells from castrated or control mice showed genes of IFN-signaling and T-helper cell pathways skewed into TH1 differentiation, including upregulation of IFNy, T-bet, and IL-12R (89). Additionally, CXCR3 expression was increased in CD4+ T cells of the castrated group suggesting suppressive effects of androgens on chemokine receptor expression relevant for tissue homing. Along this line, after castration there was an increase in CD3+ cells within lung and prostate tissue. A direct suppressive effect of testosterone on T cells was confirmed by a decrease of IFNy and T-bet expression found in splenic derived CD4+ T cells after treatment with synthetic testosterone *in vitro* (89). Further *in vitro* assays showed a reduction of STAT4 phosphorylation in CD4+ T cells upon androgen and IL-12 stimulation (89).

Confirming direct effects of androgens on mouse T cells, female T cell lines selected in the presence of DHT produced less IFNy and more IL-10 than control cell lines selected without the addition of DHT (63). Splenic derived mouse CD4+ T cells cultured with testosterone-enriched Leydig-conditioned medium showed induction of IL-10 secretion and increased Foxp3 expression, suggesting not only suppression of TH1 cytokines but also an increase in suppressor function of T cells induced by androgens (59, 63). In contrast to some previous reports of a shift toward TH2 cells, Jia et al. found reduced frequencies of TH1 and TH17 cells after *in vitro* DHT and aCD3 stimulation of mouse lymph node cells with no shift toward TH2 cells, possibly through enhanced autophagy in these cells (90). Recently, a reduction of murine *in vitro* TH1 and TH17 differentiation has been demonstrated by aromatase inhibitor treatment in combination with testosterone (91). In addition, visceral adipose tissue (VAT) from male mice showed higher Treg (CD4+ FOXP3+) frequencies then female VAT. The isolated Tregs showed differences regarding phenotype, chromatin accessibility, and transcriptional landscape. In particular, the expression of CCR2 was higher in male VAT Tregs compared to female Tregs. Female mice treated with testosterone showed an increased VAT expression of CCL2, the ligand for CCR2, and IL-6 and IL1ß, which likely stem from innate immune cells (92). These data show that the microenvironment including crosstalk with epithelial and innate immune cells clearly contributes to sex dependent differences observed in T cells.

Taken together, current knowledge suggests that androgens directly or indirectly affect T cell maturation, proliferation, and also their differentiation and cytokine production in mice and adult males. However, little is known on the direct effects of the different androgens on T cells, and specifically on the context dependent cellular and molecular mechanisms involved. Overall, androgens seem to induce a shift from TH1 effector T cells to a more suppressive phenotype. They also seem to enhance regulatory T cells. Clearly, more studies are needed that take into account signaling via classical and non-classical androgen receptors and the context dependent modulation of androgen signaling by an inflammatory microenvironment within tissues.

EFFECTS OF ANDROGENS ON T CELLS IN AUTOIMMUNITY

Autoimmune diseases (AIDs) are disorders characterized by an aberrant immune response against self-antigens. There are more than 60 different autoimmune diseases, which pose a major medical and societal challenge. The pathophysiology of most AIDs is complex and includes environmental, genetic, and epigenetic components. Most AIDs present with a strong female predisposition. MS, SLE, and the autoimmune liver disease PBC are among the diseases with the strongest female predominance (**Table 1**). While many AIDs occur more frequently in women, the course of disease may be more severe in men, exemplified by the worse disease course of male patients with MS or PBC (93, 119). Male PBC patients respond less to treatment with ursodeoxycholic acid and are at increased risk of disease progression and hepatocellular carcinoma development (119, 120). The mechanisms behind these apparent sex differences in disease susceptibility and severity are largely unknown.

PBC is a rare AID of the liver with a female to male ratio as high as 9:1 and characterized by the presence of anti-mitochondrial antibodies (AMAs), specific antinuclear antibodies (ANAs), and strong HLA associations (98, 121, 122). Immune responses directed against intrahepatic cholangiocytes, leading to the destruction and loss of small bile ducts (ductopenia) and portal inflammation with granuloma formation, are involved in the disease pathogenesis (121–123). In the other classical autoimmune liver disease, AIH, the female to male ratio is 3:1, and patients can present with elevated serum IgG-levels and/or hypergammaglobulinemia, elevated serum transaminase levels, and non-organ specific autoantibodies (101). The target cells of autoimmune attack in AIH are hepatocytes. The human leukocyte antigen alleles

TABLE 1 | Comparison of autoimmune diseases regarding female to male ratio, knowledge on testosterone serum levels, and therapeutic testosterone application.

Autoimmune disease	F:M ratio	Testosterone serum levels	Testosterone therapy	
			Human	Animal model
Multiple sclerosis (MS)	3:1 (93)	Decreased in male patients (94, 95)	Yes (94, 95)	Yes (96, 97)
Primary biliary cholangitis (PBC)	9:1 (98)	Decreased in female patients in one study (99)	No	Yes (100)
Autoimmune hepatitis (AIH)	3-4:1 (101)	Unknown	No	No (102)
Systemic lupus erythematosus (SLE)	9:1 (103, 104)	Decreased in male and female patients (105)	Yes (106, 107)	Yes (108)
Autoimmune orchitis	Male only	Unknown	No	Yes (109)
Rheumatoid arthritis (RA)	3:1 (110)	Decreased in male and female patients (111, 112)	Yes (113)	Yes (114)
Sjögren's syndrome	14:1 (115)	Decreased in female patients (116)	Yes (116)	Yes (117, 118)

(HLA)-DRB1*03:01 and HLA-DRB1*04:01 are known risk factors for AIH and may also correlate with disease course, but they are not required for AIH development (124). Many lines of evidence support the involvement of CD4+ and CD8+ T cells in both diseases' pathogenesis (122, 125). Studies have investigated the effect of sex hormones on immune cells and how sex chromosomes including X chromosome inactivation affect the sex bias in AIDs (126–130). For example, in PBC an enhanced X monosomy rate within PBMC, possibly T and B cells, compared to healthy women was found, while XCI was random and similar to the controls (131–133). Both PBC and AIH have their age peak of manifestation around menopause, and both show disease modulation by pregnancy with greatly reduced AIH activity during pregnancy and frequent flares after delivery, strongly suggesting the involvement of sex hormones (134–137). Deciphering these mechanisms may lead to novel therapeutic strategies for many of these diseases. We will focus on the studies investigating androgens in the context of autoimmunity and T cells in mouse models and in the human autoimmune liver diseases, AIH and PBC.

There are few mouse models for autoimmune liver inflammation reflecting certain aspects of autoimmune liver diseases and in some of them, a female predominance is observed similar to human disease. In a mouse model of PBC (ARE-Del$^{-/-}$), female mice showed increased serum levels of chemokines, such as MIG and IP-10, as well as increased cytokine levels including TNF, IL-10, and IL-13. They also showed increased expression of interferon Type I and II signaling in the liver compared to the male mice (138, 139). For chronic cholestatic liver inflammation and periductular fibrosis, the Mdr2$^{-/-}$ is a well-established mouse model. Already in 1997 Nieuwerk et al. described a more severe liver pathology in Mdr2$^{-/-}$ female mice compared to male mice which was associated with altered bile salt composition in bile (140). However, the impact of sex hormones on disease development in this model has not yet been investigated. We could recently identify an immunosuppressive effect of testosterone in an antigen dependent and T cell driven mouse model of experimental cholangitis. Cholangitis is induced by the transfer of antigen-specific CD8+ T cells (OT-1) which recognize their ovalbumine peptide antigen on cholangiocytes of recipient mice (100). This model shows a high female predominance. Furthermore, testosterone treatment completely suppressed liver inflammation in female mice and lack of testosterone rendered male mice susceptible to cholangitis development. Mechanistically, we could demonstrate that testosterone suppressed the expression of IL-17A by liver infiltrating lymphocytes and the hepatic expression of the lymphotropic chemokines CXCL-9 and CXCL-10 (100). Similar protective effects of testosterone were also shown in mouse models of MS and murine lupus (96, 97, 141–145). In these models, an influence of sex and androgens on the T cell expression of IFNγ and IL-10 was reported (63, 96, 97). The protective effect of testosterone on EAE development depended on androgen receptor expression and also on age, since older mice were not protected (146). In a mouse model of T cell mediated autoimmune diabetes (NOD mice), a higher *in vitro* CD4+ T cell production of IFNγ was observed in female mice and of IL-4 in male mice, which was most prominent in young NOD mice (147). In experimental autoimmune orchitis (EAO), a rat model of a male AID called autoimmune orchitis, testosterone supplementation lead to a reduced incidence of EAO (109). Testosterone treatment decreased the frequencies/numbers of CD4+ T cells and macrophages in the testis, whereas frequencies of Treg populations increased. Furthermore, testosterone treatment resulted in reduced testicular expression of TNF, IL-6, and MCP-1 (CCL2) as well as in reduced secretion of IL-2 and IFNγ of *ex vivo* stimulated mononuclear testicular lymph node cells (109).

It has been difficult to establish mouse models for AIH and few truly represent features of human disease. In one model, xenoimmunization with human antigens (Cytochrome P450 2D6 and formiminotransferase-cyclodeaminase, which are type 2 AIH self-antigens) was used, based on the principle of molecular mimicry. This model showed a higher susceptibility in females compared to males (102, 148, 149). Adoptive transfer of *ex vivo* expanded CXCR3+ Tregs recovered peripheral tolerance and ameliorated disease, but neither castration nor estradiol treatment of these mice had any effect (102, 150). To our knowledge, supplementation with testosterone or DHT was not performed to investigate the suppressive effects androgens might exert.

In humans with autoimmune liver disease, increased serum levels of the proinflammatory cytokines, IFNγ and IL-17 in AIH and PBC patients were reported, while IL-10 was lower than in healthy controls (151). TNF was reduced in the sera of these patients compared to healthy controls, but a recent publication showed an enhanced production of TNF by liver and blood derived CD4+ T cells, with a majority of these cells identified as potentially pathogenic IFNγ co-producers (151). Furthermore,

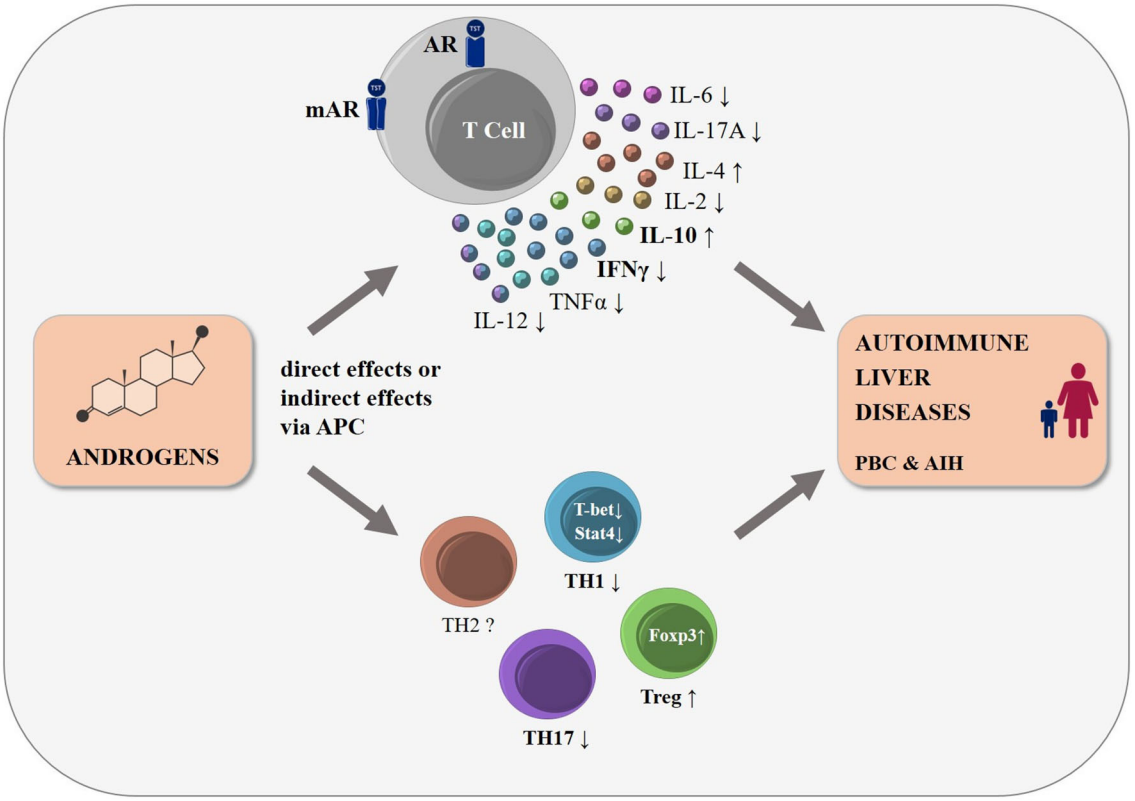

FIGURE 1 | The influence of androgens on T cell function and differentiation: schematic representation. Many autoimmune diseases, including the autoimmune liver diseases PBC and AIH, show a strong female predominance. Androgens modulate T cell development already in the thymus, mainly by altering thymic epithelial cell function (not shown). Human and mouse T cells express cytosolic (AR) and membrane bound androgen receptors (mAR). Androgens lead to changes in cytokine expression in T cells either directly or indirectly via antigen presenting cells, with a shift to a decreased pro-inflammatory cytokine expression, such as IFNγ and TNF, and an increased secretion of anti-inflammatory cytokines such as IL-10 and IL-4. Androgens were reported to reduce TH1-, TH17-, and to increase Treg-differentiation, while changes in other T cell subpopulations (e.g., TH2 cells) remain less clear. We postulate that these androgen-induced effects may influence the incidence and disease course of the T cell driven autoimmune liver diseases, PBC and AIH.

CD4+ T cells of PBC patients revealed increased expression and demethylation of CXCR3, which is the receptor for lymphotropic chemokines produced in inflamed liver (152). Although one older study showed reduced serum levels of testosterone in female PBC patients, it remains unclear whether altered sex hormone levels directly relate to some of the immunological alterations reported above (99).

Data from other AID suggest a role of testosterone in disease pathogenesis. Lower serum levels of testosterone were reported in men with MS compared to age matched healthy men, and testosterone levels seemed to correlate with disease severity (94). Another study suggested lower levels of testosterone in female MS patients compared to female age matched controls (94, 153). Of note, some pilot studies showed disease improvement upon testosterone treatment of male MS patients (94, 95, 154). Also for SLE, lower serum levels of testosterone were reported in affected women compared to age matched healthy women (105, 155, 156). The limitations of these and other studies, summarized in **Table 1**, are small cohort sizes, and they lack detailed clinical information and the use of now outdated analytical methods. Thus, studies regarding hormone levels in females with AIDs should be interpreted with caution.

Taken together, limited human data and studies using mouse models of autoimmune liver diseases hint to a higher production of proinflammatory cytokines by T cells, but a direct link to sex hormones and, specifically, androgen levels remains unclear. The novel finding of intestinal microbiota associated changes in testosterone serum levels in mice should spark interest in the role of the microbiome for sex differences in autoimmune liver diseases, which are clearly linked to an altered intestinal microbiota (157–159).

CONCLUDING REMARKS

The mechanisms behind the sex differences observed in the autoimmune liver diseases PBC and AIH, specifically the female predominance and worse disease course in male PBC patients, remain largely unknown. Emerging evidence mainly from murine studies suggests immunosuppressive effects of androgens on T cells (**Figure 1**). More studies are needed to decipher signaling pathways involved in T cells upon androgen stimulation including the classical and non-classical androgen receptors and their modulation by the local microenvironment. Understanding

the effects of androgens on immune cells may pave the way for novel treatment strategies for autoimmune liver diseases.

AUTHOR CONTRIBUTIONS

LH, DS, and CS designed and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Supraphysiological Levels of Testosterone Induce Vascular Dysfunction via Activation of the NLRP3 Inflammasome

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Background: Both supraphysiological and subphysiological testosterone levels are associated with increased cardiovascular risk. Testosterone consumption at supraphysiological doses has been linked to increased blood pressure, left ventricular hypertrophy, vascular dysfunction, and increased levels of inflammatory markers. Activation of the NLRP3 inflammasome contributes to the production of proinflammatory cytokines, leading to cardiovascular dysfunction. We hypothesized that supraphysiological levels of testosterone, via generation of mitochondrial reactive oxygen species (mROS), activates the NLRP3 inflammasome and promotes vascular dysfunction.

Methods: Male, 12 week-old C57Bl/6J (WT) and NLRP3 knockout (NLRP3$^{-/-}$) mice were used. Mice were treated with testosterone propionate [TP (10 mg/kg) *in vivo*] or vehicle for 30 days. In addition, vessels were incubated with testosterone [Testo (10^{-6} M, 2 h) *in vitro*]. Testosterone levels, blood pressure, vascular function (thoracic aortic rings), pro-caspase-1/caspase-1 and interleukin-1β (IL-1β) expression, and generation of reactive oxygen species were determined.

Results: Testosterone increased contractile responses and reduced endothelium-dependent vasodilation, both *in vivo* and *in vitro*. These effects were not observed in arteries from NLRP3$^{-/-}$ mice. Aortas of TP-treated WT mice (*in vivo*), as well as aortas from WT mice incubated with testo (*in vitro*), exhibited increased mROS levels and increased caspase-1 and IL-1β expression. These effects were not observed in arteries from NLRP3$^{-/-}$ mice. Flutamide [Flu, 10^{-5} M, androgen receptor (AR) antagonist], carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 10^{-6} M, mitochondrial uncoupler) and MCC950 (MCC950, 10^{-6} M, a NLRP3 receptor inhibitor) prevented testosterone-induced mROS generation.

Conclusion: Supraphysiological levels of testosterone induce vascular dysfunction via mROS generation and NLRP3 inflammasome activation. These events may contribute to increased cardiovascular risk.

Keywords: testosterone, androgen receptor, NLRP3 inflammasome, reactive oxygen species, vascular dysfunction

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INTRODUCTION

Epidemiological studies have shown that men are at higher risk of cardiovascular disease than women, and sex steroids seem to contribute, at least in part, to this increased risk (1, 2). Over the past five decades, preclinical studies have produced a large body of data on the molecular mechanisms involved in testosterone effects in the cardiovascular system and/or how abnormal testosterone levels modify the risk of cardiovascular disease (3, 4).

At physiological levels, testosterone induces relaxation of many vascular beds, through its influence on the production or effects of endothelium-derived relaxant factors, including nitic oxide (NO) (5, 6) and prostacyclin (7). Testosterone also releases endothelium-derived substances that cause smooth muscle cells hyperpolarization by a mechanism that involves potassium channels (8). Endothelium-independent vascular effects of testosterone, including opening of voltage-sensitive potassium channels (Kv), small- and large- conductance calcium-sensitive potassium channels (SKCa and BKCa, respectively), have also been described (9, 10). Testosterone also has an important role in the regulation of cardiac function (11, 12). It affects cardiac contractility and relaxation, and cardiomyocyte repolarization. The latter effect results in the shortening of the action potential duration. Likewise, testosterone modulates immune/inflammatory responses, displaying protective effects against atherosclerosis (13), but also stimulating *in vivo* leukocyte-endothelial cell interactions, and contributing to increased leukocyte rolling and adhesion in male spontaneously hypertensive rats (14).

In the clinical setting, patients with low plasma levels of total testosterone (<300 ng/dL) undergo hormonal treatment to improve muscle performance, bone mineral density, cognitive and sexual function as well as to prevent metabolic syndrome and cardiovascular diseases (15). The Endocrine Society Clinical Practice Guideline has recommended a dose of 75–100 mg/week of testosterone in men with hypogonadism (16). On the other hand, a survey of drug abuse in bodybuilding and weightlifting sports reported the use of anabolic androgenic steroids at doses 5– 29 times greater than the usual supplemented doses (17) to boost up muscle mass and to reduce body fat (18). Despite the beneficial effects on skeletal muscle mass and strength (19, 20), cardiac hypertrophy with sudden cardiac death is often reported among athletes and bodybuilders taking anabolic androgenic steroids (21, 22). Testosterone users show slight left ventricular hypertrophy, even after discontinuation of prolonged high testosterone administration (23). In pre-clinical studies, experimental animals treated with high doses of testosterone enanthate exhibited cardiac (left ventricle) hypertrophy, fibrosis and apoptosis (increased caspase-3, a marker of cell apoptosis) (24). In addition, testosterone in supraphysiological concentrations alters the inflammatory state with an increase in circulating levels of pro-inflammatory cytokines as tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β) and IL-12 (25, 26).

Chronic inflammation or overactivation of the immune system is a central component in the development and complications of cardiovascular diseases (27). Inflammatory responses comprise a sequence of complex interactions between immune cells, such as neutrophils, lymphocytes, monocytes/macrophages, tissue cells (including vascular and cardiac cells), and a range of inflammatory mediators such as interleukins and chemokines (28). These interactions result in increased tissue production of soluble mediators such as complement system proteins, chemokines, cytokines and eicosanoids, accompanied by increased expression of cell adhesion molecules in circulating leukocytes and endothelial cells (29).

Activation of pattern recognition receptors (PRR) in innate immune cells is a key mechanism in the genesis and progression of cardiovascular diseases (30). Members of the nucleotide-binding oligomerization domain-(NOD)-leucine-rich repeats (NLRs) receptors lead to the formation of molecular platforms called inflammasomes (31). Inflammasomes activate cysteine proteases, known as caspases, which are involved in inflammatory and apoptotic processes. The NLRP3 inflammasome, a member of the NLRP subfamily, is expressed in cardiovascular cells and its activation contributes to cardiovascular damage (32). Several endogenous components activate the NLRP3 inflammasome (33–38). Among these components, reactive oxygen species (ROS), which are produced by NLRP3 activators and are essential secondary messengers in inflammatory pathways, are involved in NLRP3 inflammasome activation (39). Based on these observations and considering that testosterone stimulates ROS generation (40, 41), we hypothesized that high levels of testosterone induce vascular dysfunction via NLRP3 inflammasome activation.

Abbreviations: ACh, acetylcholine; AR, androgen receptor; BKCa, large-conductance voltage- and calcium-activated potassium channels; BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CONCEA, National Council for Animal Experimentation Control; DHE, dihydroethidine; Emax, maximum response; Flu, flutamide; IL-12, interleukin-12; IL-1β, interleukin-1β; KCa, calcium-activated potassium channels; KCl, potassium chloride; Kv, voltage-sensitive potassium channels; MCC950, NLRP3 receptor inhibitor; mROS, mitochondrial reactive oxygen species; NADH, adenine nicotinamide dinucleotide; $NLRP3^{-/-}$, NLRP3 knockout mouse; NLRs, NOD-like receptors; NO, nitric oxide; NOD, nucleotide-binding oligomerization domain-like receptors; O_2^-, superoxide anion; PE, phenylephrine; pEC_{50}, negative logarithm of the EC_{50}; pro-IL-18, pro-interleukin-18; pro-IL-1β, pro-interlukin-1β; PRR, pattern recognition receptors; PVAT, perivascular adipose tissue; RLU, relative luminescence units; ROS, reactive oxygen species; SEM, standard error of the mean; SKCa, small-conductance voltage- and calcium-activated potassium channels; SNP, sodium nitroprusside; TBS, Tris-buffered saline; Testo, testosterone; TNFα, tumor necrosis factor alpha; TP, testosterone propionate; Vehicle, peanut oil; VSM, vascular smooth muscle; WT, wild type.

MATERIALS AND METHODS

Animals

All experimental protocols were performed in accordance with the National Council for Animal Experimentation Control and were approved by the Ethics Committee on Animal Use of the University of São Paulo, Ribeirao Preto, Brazil (Protocol n° 032/2018). Male C57BL/6J wild-type (WT) and NLRP3 knockout ($NLRP3^{-/-}$) mice (12-week-old) were obtained from the Isogenic Breeding Unit at Ribeirao Preto Medical School,

University of São Paulo, Ribeirao Preto, Brazil. Mice were maintained in a temperature (22 ± 1°C) and humidity (50–60%) controlled room on a 12-h light/dark cycle with *ad libitum* access to food and water.

WT and NLRP3$^{-/-}$ mice were treated with testosterone propionate (TP) 10 mg/kg or vehicle (peanut oil), subcutaneous injections, for 30 days. Animals were divided into four experimental groups: (1) WT_Vehicle; (2) WT_TP; (3) NLRP3$^{-/-}$_Vehicle; (4) NLRP3$^{-/-}$_TP.

Total Testosterone Levels

Plasma total testosterone levels were measured by IMMULITE 1000 Immunoassay System (Enzo Life Sciences). The samples and reagent containing the testosterone-conjugated alkaline phosphatase enzyme were distributed in 96-well plates. After 60 minutes (min) of incubation, the plates were washed to remove any remaining testosterone unbound fraction. The bound fraction was then quantified using the chemiluminescent dioxetane substrate.

Vascular Function

After isoflurane anesthesia and mice euthanasia, the thoracic aortas were removed and transferred to a modified Krebs-Henseleit solution (4°C), with the following composition [(in mM): NaCl, 130; KCl, 4.7; $NaHCO_3$, 14.9; KH_2PO_4, 1.18; $MgSO_4.7H_2O$, 1.17; Glucose 5.5; $CaCl_2.2H_2O$; 1.56; EDTA, 0.026]. Thoracic aortic rings (2 mm) were mounted on a myograph (model 620 M; Danish Myo Technology – DMT, Copenhagen, Denmark) containing Krebs-Henseleit solution gassed with 5% CO_2/95% O_2 to maintain a pH of 7.4 for isometric tension recording. After the stabilization period, arteries were stimulated with potassium chloride (120 mM KCl) to verify functional integrity. Endothelial integrity was confirmed by over 80% relaxation to acetylcholine [(ACh), endothelium-dependent vasodilator, 10^{-6} M] on vessels pre-contracted with phenylephrine [(PE), alpha-adrenergic agonist, 10^{-6} M]. All the aortic rings used in this study presented intact endothelium.

Cumulative concentration-effect curves for PE (10^{-10}-10^{-4} M), ACh (10^{-10}-10^{-4} M) and sodium nitroprusside [SNP (10^{-10}-10^{-4} M)] were performed in all experimental groups. The *in vitro* effects of testosterone [incubation of vessels with testosterone 10^{-6} M, for 2 hours (h)] on NLRP3 inflammasome activation, mitochondrial ROS generation and androgen receptor (AR) activation were evaluated using a NLRP3 receptor inhibitor (MCC950, 10^{-6} M for 30 min), a mitochondrial oxidative phosphorylation uncoupler [carbonyl cyanide 3-chlorophenyl hydrazone (CCCP), 10^{-6} M for 30 min] and an androgen receptor antagonist [Flutamide (Flu), 10^{-5} M, for 30 min], respectively. To verify the *in vivo* effects of supraphysiological levels of testosterone on vascular function, we used thoracic aortic segments from mice treated with testosterone propionate [TP (10 mg/kg for 30 days)].

Determination of Cytokine Levels

IL-1β was quantified in the serum and thoracic aortas by Enzyme-Linked ImmunonoSorbent Assay [(ELISA) R&D Systems, MLB00C], which is based on antigen-antibody reactions detectable by enzymatic reactions.

Measurement of Reactive Oxygen Species

Dihydroethidine

ROS generation was determined using a qualitative method involving dihydroethidine (DHE), a non-fluorescent precursor to ethidium bromide, as previously described by Suzuki et al. (42). In the presence of ROS, dihydroethidine is oxidized inside the cell, producing the fluorescent compounds ethid (E) and 2-hydroxy ethid (EHO), which have an affinity for nuclear DNA. Aortas of WT and NLRP3$^{-/-}$ mice treated with TP or vehicle were isolated and quickly immersed in freezing medium. Using a cryostat (Leica, Germany), cross sections of the aorta (5 μm) were obtained and placed on silanized slides. The sections were incubated with DHE (5×10^{-6} M) for 30 min at 37°C in a humid chamber protected from light. After this period, the slides were observed in an optical microscope (ZEISS) equipped with a rhodamine filter and a photographic camera, using a fluorescence microscopy. ROS generation was quantified through the light density corrected by the area using the program ImageJ (National Institutes of Health).

Lucigenin

Superoxide anion (O_2^-) generation in thoracic aortas was measured by chemiluminescence assay. The adenine nicotinamide dinucleotide (NADH) which is expected to potentiate O_2^- production by the respiratory chain via complex I, was used as the substrate (43). Aortas were incubated with testosterone [Testo (10^{-6} M) for 2 h] in the absence or presence of Flu (10^{-5} M) for 30 min, CCCP (10^{-6} M) for 30 min and MCC950 (10^{-6} M) for 30 min, which were added before the incubation with Testo. Aortas were then transferred into glass tubes containing 990 μL assay buffer (50 mM KH_2PO_4, 1 mM EGTA and 150 mM sucrose, pH 7.4) and 5 μL of lucigenin (5×10^{-6} M) for basal reading. After the baseline reading, 10 μL of NADH (10^{-6} M) was added. The Line TL Tube Luminometer (Titertek-Berthold®, Pforzheim, Germany) was used for quantification of superoxide anion generation and data were expressed in relative luminescence units (RLU)/protein concentration (mg).

Western Blotting

Protein expression of NLRP3 and caspase-1 was determined using protein analysis of thoracic aortas of *in vivo* and *in vitro* all groups. Samples were homogenized in lysis buffer and proteins were collected. Proteins (30 μg) were separated by electrophoresis on 10 or 12% polyacrylamide gels, transferred to 0.22 μm nitrocellulose membranes and blocked using 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) and 0.1% Tween 20 for 1 h. Primary antibodies were incubated overnight at 4°C as follows: anti-NLRP3 (1:500 dilution; R&D Systems), anti-caspase-1 (1:1,000 dilution; Novus Biologicals), anti-β-actin-peroxidase (1:5,000 dilution; Sigma-Aldrich).

TABLE 1 | Characteristics of WT and NLRP3$^{-/-}$ mice treated with TP or Vehicle.

	WT_Vehicle	WT_TP	NLRP3$^{-/-}$_Vehicle	NLRP3$^{-/-}$_TP
Testosterone (ng/dL)	548.08 ± 205.45	2829.83 ± 302.68*	460.0 ± 73.41	2803.33 ± 330.3*
Body mass (g)	24.00 ± 1.06	31.62 ± 0.55*	24.14 ± 0.32	28.65 ± 0.73*
Epididymal fat (g)	0.404 ± 0.005	0.188 ± 0.011*	0.341 ± 0.011	0.190 ± 0.004*
MAP (mmHg)	99.85 ± 4.03	127.26 ± 3.31*	113.90 ± 2.58	108.53 ± 2.76#

*Data represent the mean ± S.E.M (n = 5–10 mice per group). Two-way ANOVA: *p < 0.05 vs. respective Vehicle group; #p < 0.05 vs. WT_TP. MAP, mean arterial pressure; TP, testosterone propionate; WT, wild type.*

Blood Pressure

Mice were anesthetized with a mixture of isoflurane 2% and O_2 for carotid artery cannulation. Subcutaneous administration of tramadol (12.5 mg/kg) in a single dose was performed to promote postoperative analgesia. Twenty-four hours later the catheter was coupled to a pressure transducer connected to an amplification system and a computer with an analog-to-digital interface (PowerLab/4SP, ADInstruments, Colorado Springs, CO). After stabilization, blood pressure was determined.

Drugs

Testosterone propionate, phenylephrine, acetylcholine, sodium nitroprusside, testosterone, flutamide, were purchased from Sigma Chemical Co (St. Louis, MO, USA), CCCP (Tocris®, Bristol, United Kingdom) and MCC950 (Avistron®, Bude, Cornwall, United Kingdom).

Statistical Analysis

For analysis of vascular reactivity, individual concentration-effect curves were plotted on a sigmoidal curve, by non-linear regression analysis. These curves, in turn, provide the maximum response value (Emax) and pEC_{50} (negative logarithm of the EC_{50} values - concentration that produces 50% of the maximum response). The values of Emax and pEC_{50} were compared using Student's *t*-test and two-way analysis of variance test (Two-way ANOVA), followed by the Tukey post-test. The results of the molecular experiments were analyzed by Student's *t*-test and Two-way ANOVA, followed by the Tukey post-test. Data were assessed for normality with Shapiro-Wilk test. Potential outliers were analyzed using the GraphPad Prism Outlier Test. In the present study, no outliers or data were excluded. The program GraphPad Prism, version 6.0 (GraphPad Software Inc., San. Diego, CA, USA) was used to analyze these parameters. The results were expressed as mean ± standard error of the mean (SEM). The acceptable level of significance was $p < 0.05$.

RESULTS

Deletion of NLRP3 Prevents *in vivo* Effects of Supraphysiological Levels of Testosterone on Vascular Function

To address whether supraphysiological levels of testosterone promote vascular dysfunction via NLRP3 inflammasome, WT and NLRP3$^{-/-}$ mice were treated with TP (10 mg/kg for 30 days) or vehicle. TP treatment increased total testosterone plasma levels in both experimental groups. Supraphysiological levels of testosterone in the TP groups were confirmed by comparison with vehicle-treated mice. Testosterone also increased body mass and decreased epididymal fat in WT and NLRP3$^{-/-}$ mice in comparison to vehicle-treated mice. On the other hand, only TP-treated WT mice exhibited increased blood pressure (**Table 1**).

Aortic rings from TP-treated WT mice exhibited increased PE-induced contractile responses compared to aortas from vehicle-treated WT mice (**Figure 1A** and **Table 2**). In addition, ACh-mediated endothelium-dependent vasodilation was decreased in aortic rings of TP-treated WT mice compared to those from vehicle-treated WT mice (**Figure 1B** and **Table 2**). Deletion of NLRP3 prevented testosterone-induced increased contractile responses to PE (**Figure 1A** and **Table 2**) as well as impaired ACh vasodilation (**Figure 1B** and **Table 2**). Aortas from TP-treated WT mice did not exhibit altered responses to SNP (**Table 2**).

Activation of the NLRP3 inflammasome by testosterone was then evaluated. *In vivo*, treatment of WT mice with TP increased NLRP3 expression (**Figure 1C**) and caspase-1 activation (**Figure 1D**) in thoracic aortas. Serum IL-1β levels were increased in TP-treated WT mice (**Figure 1E**). Lack of NLRP3 prevented increased caspase-1 activation and IL-1β release in TP-treated mice (**Figures 1D,E**).

Vascular Dysfunction Induced by *in vitro* Treatment of Aortic Rings With Testosterone Involves NLRP3 Inflammasome Activation

Mechanisms by which NLRP3 inflammasome contributes to testosterone-induced vascular dysfunction were investigated in aortic rings, from WT and NLRP3$^{-/-}$ mice, incubated with testosterone (10^{-6} M for 2 h). PE induced concentration-dependent contractions that were increased in testosterone-treated aortic rings compared to vehicle-treated rings (**Figure 2A** and **Table 3**). In addition, ACh-mediated vasodilation was decreased in aortic rings incubated with testosterone compared to vehicle-treated aortas (**Figure 2B** and **Table 3**). Lack of NLRP3 receptor completely prevented testosterone-induced increased contractile responses to PE (**Figure 2A** and **Table 3**) and impaired ACh-induced vasodilation (**Figure 2B** and **Table 3**). Testosterone did not alter vasodilator responses to SNP (**Table 3**). Activation of the NLRP3 inflammasome was also evaluated in these vessels. *In vitro*, testosterone increased NLRP3 expression (**Figure 2C**), caspase-1 activation (**Figure 2D**) and

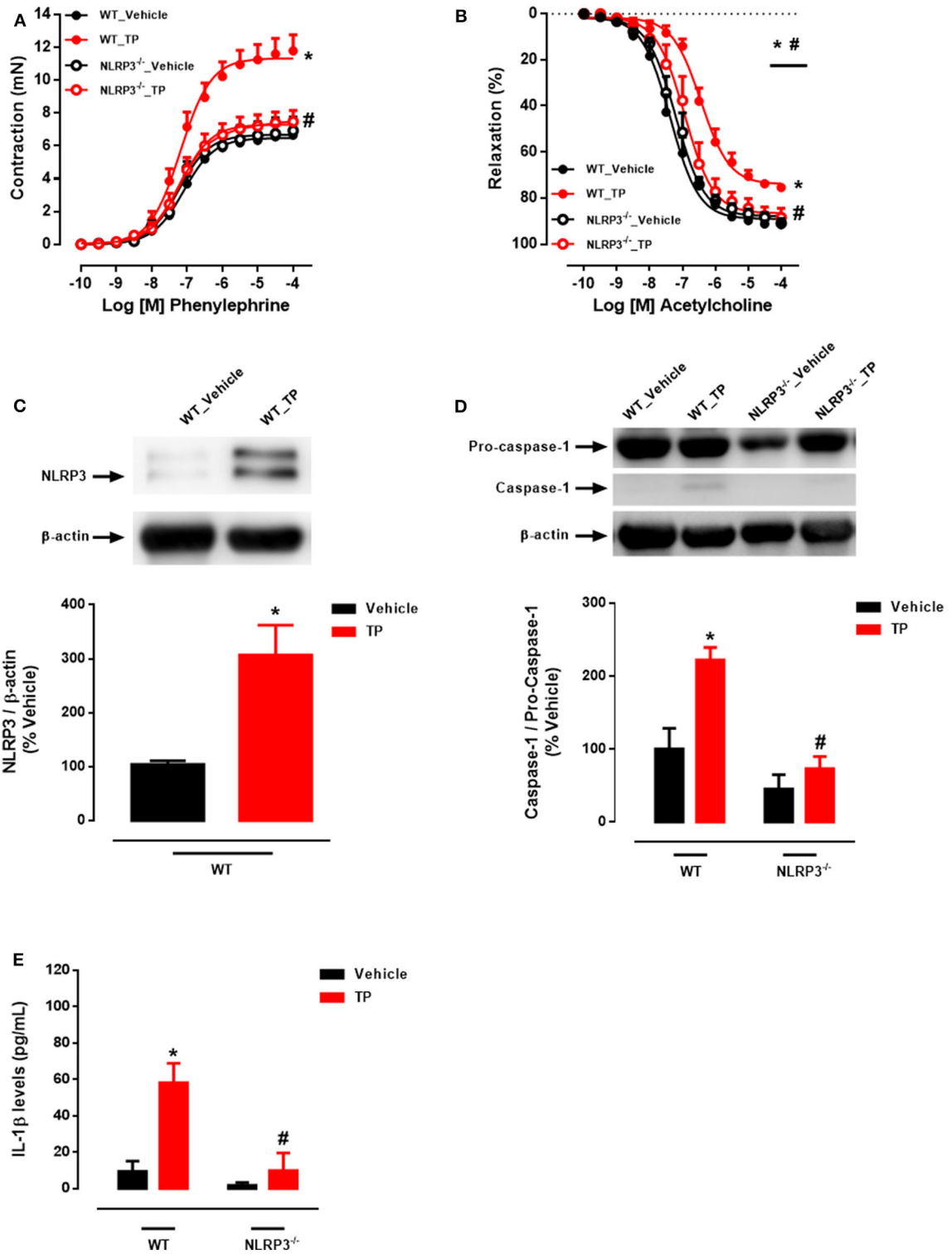

FIGURE 1 | Testosterone propionate treatment induces vascular dysfunction via NLRP3 inflammasome (*in vivo* experiments). Concentration-response curves to phenylephrine - PE **(A)** and acetylcholine - ACh **(B)** were performed in aortic rings; NLRP3 **(C)** and caspase-1 **(D)** expression was determined in thoracic aortas; IL-1β levels **(E)** in the serum of WT and NLRP3$^{-/-}$ mice treated with testosterone propionate (10 mg/Kg for 30 days). Data are expressed as mean ± SEM ($n = 3$–10). *$p < 0.05$ vs. WT_Vehicle; #$p < 0.05$ vs. WT_TP.

TABLE 2 | Maximal response and pEC_{50} values for PE-induced contraction and ACh- and SNP-induced relaxation in aortas of WT and NLRP3$^{-/-}$ mice treated with TP or Vehicle.

	PE		ACh		SNP	
	Emax (mN)	pEC_{50}	Emax (%)	pEC_{50}	Emax (%)	pEC_{50}
WT_Vehicle	6.47 ± 0.32	7.1 ± 0.15	89.04 ± 1.4	7.37 ± 0.06	95.69 ± 3.4	7.53 ± 0.11
WT_TP	11.33 ± 0.31*	7.2 ± 0.09	73.98 ± 1.74*	6.44 ± 0.06*	97.58 ± 2.69	7.12 ± 0.07
NLPR3$^{-/-}$_Vehicle	6.67 ± 0.19	7.2 ± 0.09	87.78 ± 1.55	7.20 ± 0.06	–	–
NLRP3$^{-/-}$_TP	7.29 ± 0.24$^{\#}$	7.2 ± 0.1	86.58 ± 2.62$^{\#}$	6.93 ± 0.09$^{\#}$	–	–

*Data represent the mean ± S.E.M (n = 5–7 mice per group). Two-way ANOVA: *p < 0.05 vs. WT_Vehicle; #p < 0.05 vs. WT_TP. Emax, maximal response; pEC_{50}, negative logarithm of the EC_{50}; PE, phenylephrine; ACh, acetylcholine; SNP, sodium nitroprusside; TP, testosterone propionate; WT, wild type.*

IL-1β levels (**Figure 2E**) expression in thoracic aortas. Aortas from NLRP3$^{-/-}$ mice did not exhibit increased caspase-1 activation (**Figure 2D**) or IL-1β expression (**Figure 2E**) in response to testosterone.

Pharmacological Inhibition of NLRP3 Inflammasome, Androgen Receptors and ROS Prevents Testosterone-Induced Vascular Dysfunction

The contribution of the NLRP3 inflammasome to testosterone-induced vascular dysfunction was further evaluated using a selective NLRP3 inhibitor, MCC950. MCC950 prevented the increased contractile responses to PE (**Figure 3A** and **Table 4**) and partially prevented the impairment of ACh-induced vasodilation (**Figure 3B** and **Table 4**). No differences were observed between reactivity of MCC950- and vehicle-treated aortas of WT mice.

To determine whether AR mediate testosterone-induced vascular dysfunction, Flu, an AR antagonist, was used. Flu prevented testosterone-induced increased contractile responses to PE (**Figure 3C** and **Table 5**) and the impaired ACh vasodilation (**Figure 3D** and **Table 5**). No differences were observed between vascular reactivity of thoracic aortic rings of WT mice incubated with Flu and vehicle.

Supraphysiological Testosterone Levels Induce Vascular Generation of Mitochondria-Derived Reactive Oxygen Species

Initially, ROS generation in thoracic aortas of WT and NLRP3$^{-/-}$ mice treated with TP or vehicle was determined. TP treatment increased ROS vascular generation in WT mice, but not in NLRP3$^{-/-}$ mice (**Figure 4A**).

Mitochondrial ROS generation was also determined in thoracic aortas of WT and NLRP3$^{-/-}$ mice incubated with testosterone or vehicle. Testosterone increased mROS in aortas from WT mice, but not in NLRP3$^{-/-}$ aortas (**Figure 4B**). The contribution of NLRP3 inflammasome (**Figure 4C**), AR (**Figure 4D**) and mitochondrial electron transport chain (**Figure 4E**) to ROS generation was also determined *in vitro* in aortas from WT mice. Mitochondrial uncoupling by CCCP, an inhibitor of oxidative phosphorylation, NLRP3 deletion and blockade of AR abrogated testosterone-induced vascular ROS generation.

To determine whether mitochondrial ROS contribute to the vascular effects of testosterone, experiments were performed in the presence of the mitochondrial uncoupler CCCP. CCCP prevented testosterone-induced increased in contractile responses to PE (**Figure 5A** and **Table 6**) and the impaired ACh vasodilation (**Figure 5B** and **Table 6**). No differences were observed between vascular reactivity of thoracic aortic rings of WT mice treated with CCCP and vehicle.

DISCUSSION

The primary goal of the present study was to uncover whether supraphysiological levels of testosterone induce NLRP3 inflammasome activation and consequent vascular dysfunction. *In vitro* studies using thoracic aortas and *in vivo* treatment of WT and NLRP3$^{-/-}$ mice demonstrated that supraphysiological levels of testosterone activate the NLRP3 inflammasome in vascular cells and cause vascular dysfunction. Our study provides *in vivo* evidence that NLRP3 inflammasome activation mediates proinflammatory effects of elevated levels of testosterone, thereby contributing to vascular dysfunction. Antagonism of testosterone receptors and inhibition of mitochondrial ROS generation prevent testosterone-induced vascular dysfunction *in vitro*. These findings, for the first time, demonstrate a key role for both increased ROS-mediated signaling and activation of NLRP3 inflammasome to vascular damage induced by testosterone.

Whereas, the consequences of testosterone deprivation in the cardiovascular system have been investigated (44), the effects of supraphysiological levels of the hormone have been surprisingly little studied. Evidence indicates that supraphysiological levels of testosterone affect the function and structure of the cardiovascular system. Sprague-Dawley rats treated with supraphysiological doses of testosterone show eccentric cardiac hypertrophy mediated by ERK 1/2 and mTOR phosphorylation (45). Increased systemic testosterone also decreases relaxation of human pulmonary arteries and veins (46).

In the present study, we addressed direct and long-term effects of testosterone on NLRP3 inflammasome-mediated

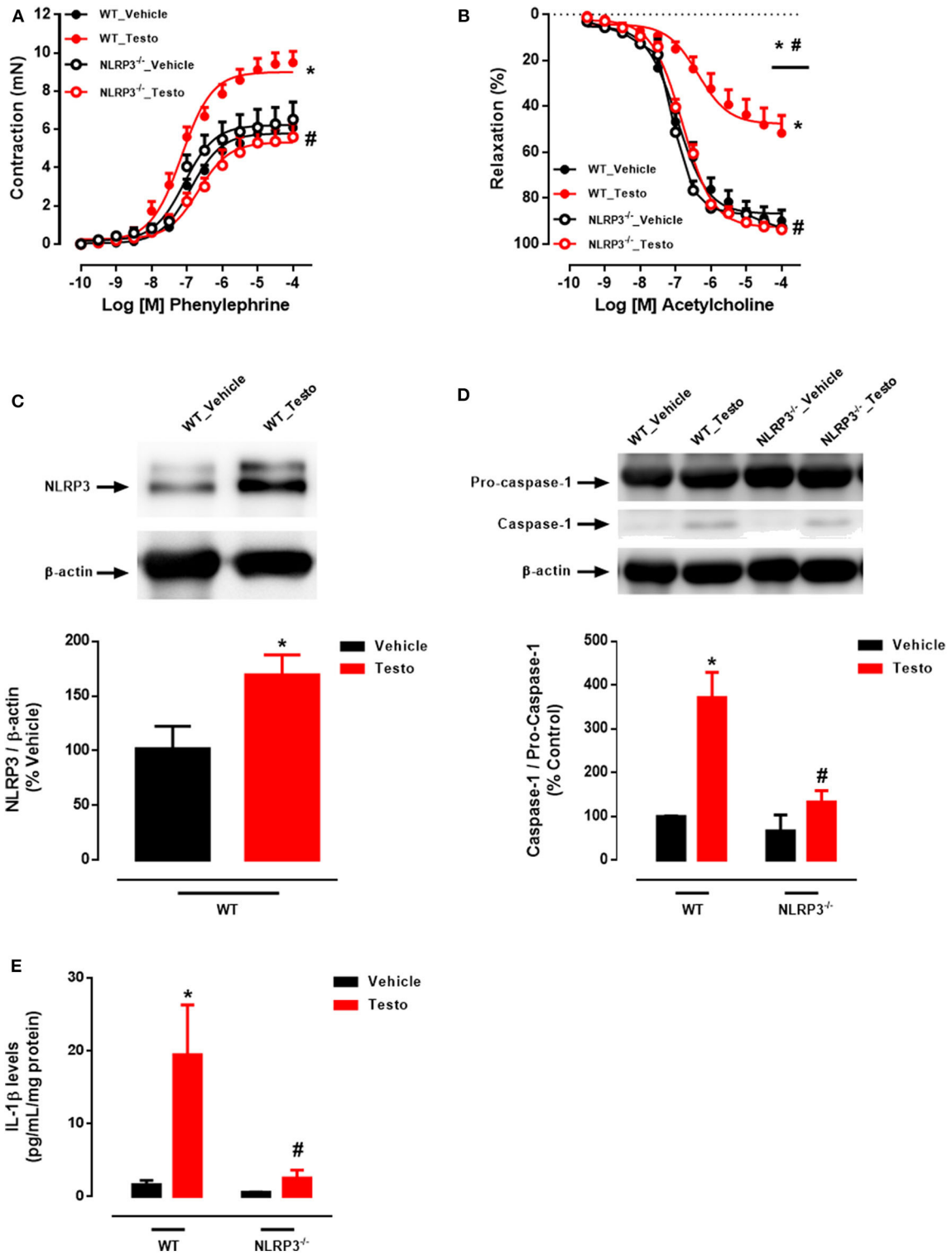

FIGURE 2 | Testosterone induces vascular dysfunction via NLRP3 inflammasome (*in vitro* experiments). Concentration-response curves to phenylephrine - PE **(A)** and acetylcholine - ACh **(B)**; NLRP3 **(C)**, caspase-1 **(D)** and IL-1β levels **(E)** expression, all determined in thoracic aortas incubated with testosterone (10^{-6} M for 2 h) from WT and NLRP3$^{-/-}$ mice. Data are expressed as mean ± SEM ($n = 3$–10). $^{*}p < 0.05$ vs. WT_Vehicle; $^{\#}p < 0.05$ vs. WT_Testo.

TABLE 3 | Maximal response and pEC_{50} values for PE-induced contraction and ACh- and SNP-induced relaxation in aortas from WT and $NLRP3^{-/-}$ mice stimulated *in vitro* with Testo or Vehicle.

	PE		ACh		SNP	
	Emax (mN)	**pEC_{50}**	**Emax (%)**	**pEC_{50}**	**Emax (%)**	**pEC_{50}**
WT_Vehicle	5.79 ± 0.15	6.89 ± 0.08	86.79 ± 2.0	6.95 ± 0.08	95.69 ± 1.61	8.15 ± 0.06
WT_Testo	9.0 ± 0.21*	7.15 ± 0.08	47.67 ± 2.68*	6.36 ± 0.16*	99.56 ± 1.77	8.23 ± 0.07
$NLPR3^{-/-}$_Vehicle	6.22 ± 0.29	7.08 ± 0.15	92.66 ± 1.09	6.8 ± 0.04	–	–
$NLRP3^{-/-}$_Testo	5.32 ± 0.24#	6.67 ± 0.13#	90.9 ± 1.56#	7.05 ± 0.06#	–	–

*Data represent the mean ± S.E.M (n = 4–10 mice per group). Two-way ANOVA: *p < 0.05 vs. WT_Vehicle; #p < 0.05 vs. WT_Testo. Emax, maximal response; pEC_{50}, negative logarithm of the EC_{50}; PE, phenylephrine; ACh, acetylcholine; SNP, sodium nitroprusside; Testo, testosterone; WT, wild type.*

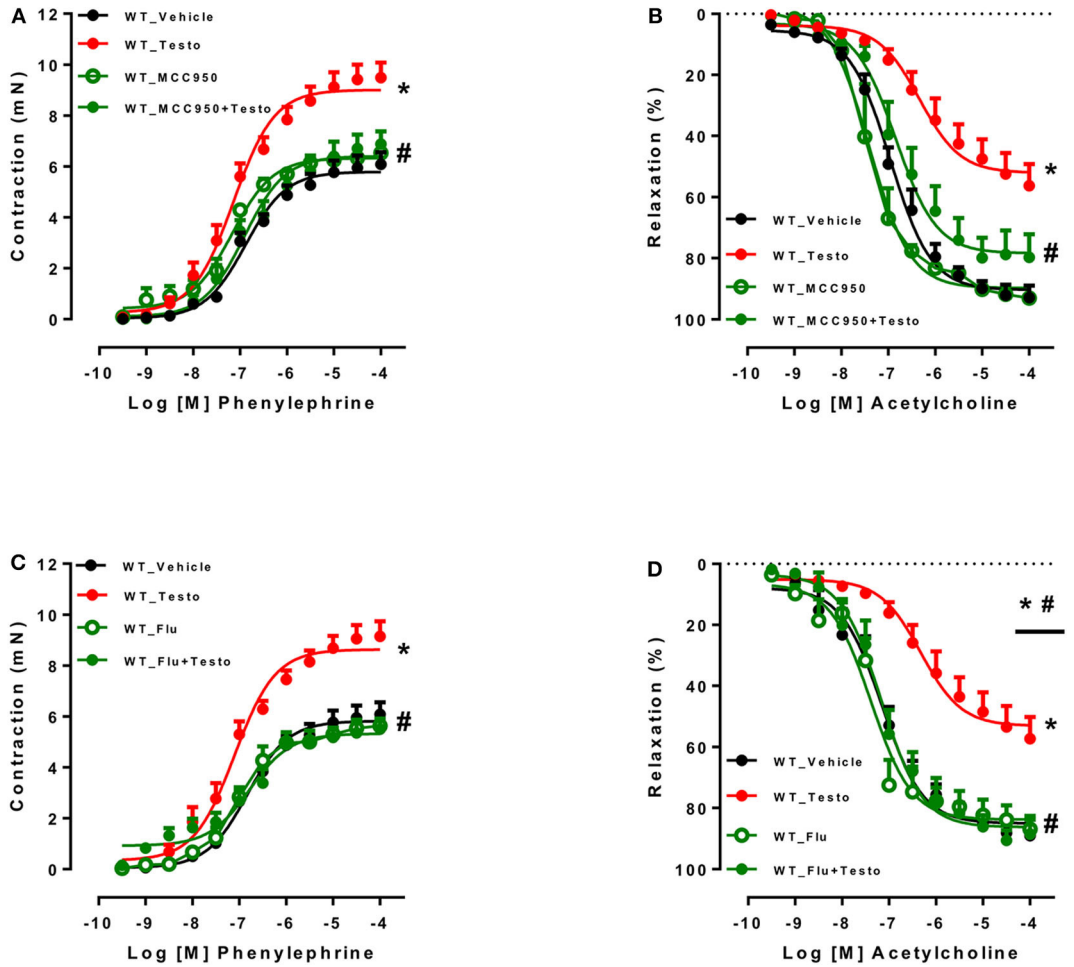

FIGURE 3 | Pharmacological inhibition of NLRP3 inflammasome and androgen receptor prevents testosterone-induced vascular dysfunction. Concentration-response curves to phenylephrine - PE **(A,C)** and acetylcholine - ACh **(B,D)** were performed in aortic rings incubated with vehicle (WT_Vehicle) or testosterone (10^{-6} M for 2 h) (WT_Testo). The effects of MCC950 (10^{-6} M for 30 min) and Flutamide [Flu (10^{-5} M for 30 min)] on testosterone-induced vascular changes are shown in **(A,B)** - WT_MCC950 and WT_MCC950+Testo - and **(C,D)** - WT_Flu and WT_Flu+Testo - respectively. Data are expressed as mean ± SEM (n = 3–10). *p < 0.05 vs. WT_Vehicle; #p < 0.05 vs. WT_Testo.

vascular dysfunction. The innate and adaptive immune responses significantly influence both acute and chronic changes in cardiovascular phenotype that lead to clinical cardiovascular abnormalities (47–50).

Members of the NLR family have emerged as important sensors involved in the immune responses to pathogens and inflammatory diseases. NLRP3, a well-characterized member of the NLR family, regulates the assembly of the inflammasome, a

TABLE 4 | Maximal response and pEC_{50} values for PE-induced contraction and ACh-induced relaxation in aortas incubated with Testo, MCC950, MCC950+Testo or Vehicle from WT mice.

	PE		Ach	
	Emax (mN)	**pEC_{50}**	**Emax (%)**	**pEC_{50}**
WT_Vehicle	5.79 ± 0.15	6.89 ± 0.08	90.38 ± 1.78	6.96 ± 0.06
WT_Testo	9.0 ± 0.22*	7.14 ± 0.08	52.05 ± 2.67*	6.34 ± 0.15
WT_MCC950	6.3 ± 0.13	6.9± 0.07	88.76 ± 3.06	7.41 ± 2.15
WT_MCC950+Testo	6.4 ± 0.22#	7.17 ± 0.10	78.38 ± 3.0*	6.89 ± 0.12

*Data represent the mean ± S.E.M (n = 3–10 mice per group). Two-way ANOVA: *p < 0.05 vs. WT_Vehicle; #p < 0.05 vs. WT_Testo. Emax, maximal response; pEC_{50}, negative logarithm of the EC_{50}; PE, phenylephrine; ACh, acetylcholine; Testo, testosterone; MCC950, NLRP3 antagonist; WT, wild type.*

TABLE 5 | Maximal response and pEC_{50} values for PE-induced contraction and ACh-induced relaxation in aortas incubated with Testo, Flu, Flu+Testo or Vehicle from WT mice.

	PE		Ach	
	Emax (mN)	**pEC_{50}**	**Emax (%)**	**pEC_{50}**
WT_Vehicle	5.82 ± 0.16	6.83 ± 0.08	85.0 ± 2.2	7.15 ± 0.09
WT_Testo	8.63 ± 0.21*	7.1 ± 0.08	53.1 ± 2.67*	6.32 ± 0.14*
WT_Flu	5.44 ± 0.12	7.01 ± 0.07	83.76 ± 2.4	7.38 ± 0.12
WT_Flu+Testo	5.32 ± 0.23#	6.78 ± 0.12	86.72 ± 2.5#	7.16 ± 0.09#

*Data represent the mean ± S.E.M (n = 3–10 mice per group). Two-way ANOVA: *p < 0.05 vs. WT_Vehicle; #p < 0.05 vs. WT_Testo. Emax, maximal response; pEC_{50}, negative logarithm of the EC_{50}; PE, phenylephrine; ACh, acetylcholine; Testo, testosterone; Flu, Flutamide, androgen receptor antagonist; WT, wild type.*

multimeric complex protein that activates inflammatory caspase-1, which cleaves pro-interleukin-1β (pro-IL-1β) and pro-IL-18 into their mature and biologically active forms (51). Vascular cells can detect and respond to a variety of signals that are indicative of cell damage, including environmental irritants, endogenous danger signals, pathogens, and mitochondria-derived ROS, leading to the release of cytokines, chemokines and hormones (52, 53). The present study supports that the NLRP3 inflammasome is involved in the vascular dysfunction triggered by high testosterone levels. First, NLRP3 gene deletion prevented testosterone-induced hypercontractility of vascular smooth muscle (VSM) and endothelial dysfunction both *in vitro* and *in vivo*. Vascular alterations in response to testosterone *in vitro* were also partially prevented in the presence of MCC950, further indicating that NLRP3 inflammasome is key to vascular damage induced by testosterone. The observations that both chronic treatment of mice with testosterone propionate and incubation of aortas with testosterone increase vascular caspase-1 expression as well as IL-1β levels provide further support to the idea that testosterone induces vascular NLRP3 inflammasome activation.

Our group recently demonstrated that NLRP3 receptor inhibition restores the functional integrity of resistance mesenteric arteries of diabetic animals (38). Previous studies also showed that IL-1β, a key immunoregulatory and proinflammatory cytokine produced by the inflammasome, reduces endothelium-dependent relaxation, increases vascular contractility, as well as VSM cells migration and proliferation with consequent vascular remodeling (37, 54–56).

ROS production, especially from the mitochondria, triggers NLRP3 inflammasome activation (57). Testosterone induces long-term ROS production by genomic mechanisms in a time- and concentration-dependent manner. Also, it stimulates short-term ROS production by unique nongenomic mechanisms in VSM cells from hypertensive animals (40). In that the present study *in vitro* treatment of vascular segments with testosterone increased vascular ROS generation. Interestingly, chronic treatment with TP and incubation of arteries with testosterone increased IL-1β, the end product of NLRP3 inflammasome activation (58). Finally, the findings that vascular ROS generation in response to testosterone incubation is prevented by MCC950, Flu and CCCP, imply AR and mitochondria on testosterone-induced ROS generation in VSM cells (41).

To determine NLRP3 inflammasome activation in testosterone-induced ROS generation and vascular dysfunction *in vivo*, we used $NLRP3^{-/-}$ mice treated with TP. Our analysis revealed that inflammasome signaling is critical to testosterone-induced vascular dysfunction. Lack of NLRP3 inflammasome abolished testosterone-induced ROS generation and IL-1β production, indicating an interplay between ROS and NLRP3, as trigger and effector molecules.

The role of mitochondrial ROS in the vascular effects of high testosterone concentrations were further investigated in our study. CCCP prevented the deleterious effects of testosterone on vascular function. This is consistent with earlier data from our group, showing that mitochondrial ROS from the perivascular adipose tissue (PVAT) of obese mice induces vascular dysfunction (59).

AR modulate inflammatory processes and activation of components of the immune system. Accordingly, male mice lacking AR in monocytes/macrophages exhibit less eosinophil recruitment and lung inflammation due to impaired M2 polarization (60). On the other hand, animals with deletion of AR in macrophages/monocytes are protected from atherosclerosis (61) and depletion of AR has protective effects on abdominal aortic aneurysm development in WT mice (62). In this study Flu prevented testosterone effects on vascular function and ROS generation, supporting that AR contribute to testosterone-induced activation of inflammasome and oxidative stress. In addition to inhibition of mitochondrial ROS generation and NLRP3 inflammasome activation, blockade of AR prevented impaired vascular contractile and relaxant responses. The evidence that testosterone, via AR, promotes calcium (Ca^{2+}) influx (63) and that intracellular Ca^{2+} is crucial for the generation of mitochondrial ROS (64) is consistent with our hypothesis that supraphysiological levels of testosterone induces vascular dysfunction via increased mitochondrial ROS-mediated activation of NLRP3 inflammasome. Since testosterone activates other receptors, including GPRC6A and GPER (65), further studies are warranted for a more detailed investigation on the relative contribution of AR to the vascular complications induced by supraphysiological concentrations of testosterone.

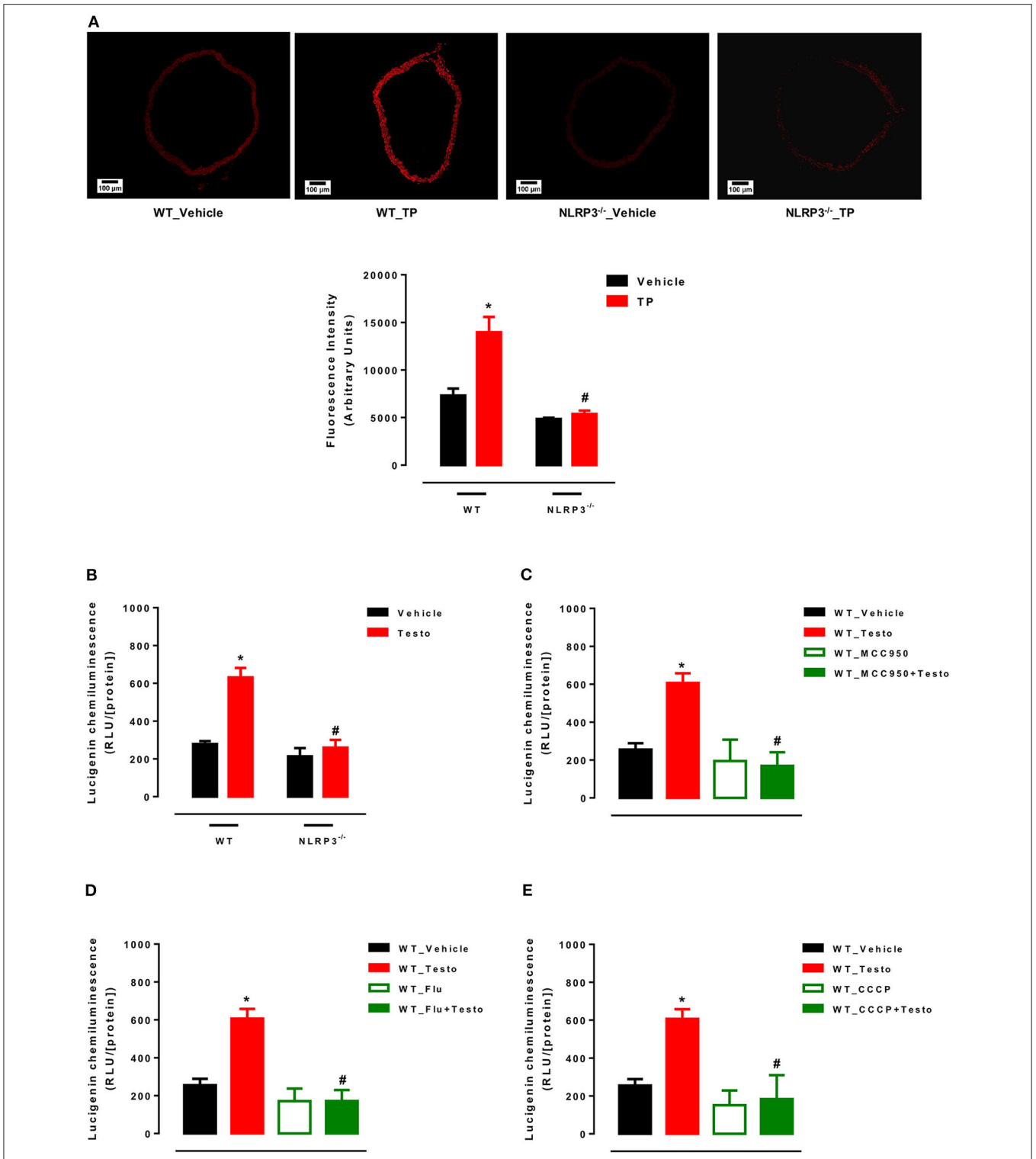

FIGURE 4 | Supraphysiological testosterone levels induce vascular generation of reactive oxygen species. Vascular ROS generation was measured by dihydroethidine in aortas from WT_Vehicle, WT_TP, NLRP3$^{-/-}$_Vehicle and NLRP3$^{-/-}$_TP mice **(A)**; superoxide anion generation – measured by lucigenin in vessels incubated with Testo or vehicle (WT_Vehicle, WT_Testo, NLRP3$^{-/-}$_Vehicle and NLRP3$^{-/-}$_Testo) **(B)**; effects of MCC950 **(C)**, Flu **(D)** and CCCP **(E)** on testosterone effects in aortas isolated from WT mice and incubated with vehicle or testosterone. Data are expressed as mean ± SEM ($n = 5$). *$p < 0.05$ vs. WT_Vehicle; $^{\#}p < 0.05$ vs. WT_TP; $^{\#}p < 0.05$ vs. WT_Testo.

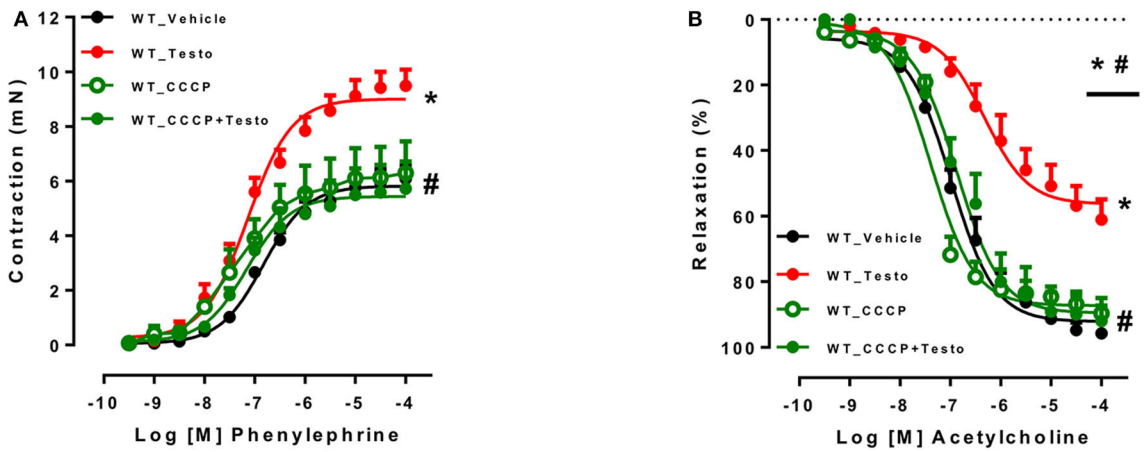

FIGURE 5 | Testosterone induces vascular dysfunction via the generation of mitochondrial reactive oxygen species. Concentration-response curves to phenylephrine - PE **(A)** and acetylcholine - ACh **(B)** were performed in aortic rings incubated with testosterone (10^{-6} M for 2 h) or vehicle isolated from WT mice. The effects of CCCP (10^{-6} M for 30 min) on testosterone effects were determined. Data are expressed as mean ± SEM ($n = 3$–10). $^{*}p < 0.05$ vs. WT_Vehicle; $^{\#}p < 0.05$ vs. WT_Testo.

TABLE 6 | Maximal response and pEC_{50} values of PE-induced contraction and ACh-induced relaxation in aortas incubated with Testo, CCCP, CCCP+Testo or Vehicle from WT mice.

	PE		Ach	
	Emax (mN)	**pEC_{50}**	**Emax (%)**	**pEC_{50}**
WT_Vehicle	5.82 ± 0.16	6.83 ± 0.08	92.14 ± 1.7	6.99 ± 0,06
WT_Testo	9.0 ± 0.22*	7.14 ± 0.08	56.3 ± 2.7*	6.32 ± 0.13*
WT_CCCP	6.03 ± 0.33	7.28 ± 0.22	87.3 ± 2.2	7.4 ± 0.11
WT_CCCP+Testo	5.44 ± 0.3#	7.16 ± 0.18	89.5 ± 2.8#	6.88 ± 0.10#

Data represent the mean ± S.E.M ($n = 3$–10 mice per group). Two-way ANOVA: $^{*}p < 0.05$ vs. WT_Vehicle; $^{\#}p < 0.05$ vs. WT_Testo. Emax, maximal response; pEC_{50}, negative logarithm of the EC_{50}; PE, phenylephrine; ACh, acetylcholine; Testo, testosterone; CCCP, carbonyl cyanide m-chlorophenyl hydrazone, inhibitor of oxidative phosphorylation; WT, wild type.

Meanwhile, our study highlights classical AR in the pathogenesis of vascular dysfunction.

The current study has strengths and limitations. The experiments rigorously followed stringent quality criteria in experimental research such as randomization, manipulation and evaluations performed in a blinded fashion, controlled physiological parameters and use of control groups of health animals. A major limitation of the current study was the *in vivo* approach, which restricts mechanistic investigations of tissue-specific inflammatory pathways, since it does not allow identification of the specific cell types responsible for the observed systemic changes. Considering that the primary goal of this study was to directly investigate mechanisms of impaired vascular dysfunction induced by high systemic levels of testosterone, to overcome this limitation we also addressed the effects of testosterone on isolated vessels. The analysis of molecular mechanisms involved in testosterone-induced activation of NLRP3 was supported by the *in vitro* approaches. However, results observed on *in vitro* assays, although important, often fail to translate to similar results *in vivo*. Additionally, although DHE has been extensively used DHE to detect O_2^- in cells or systems, the interference from other oxidative radicals prevents a fine tune quantification of O_2^- (66, 67). Since every method has shortcomings, we used a combination of DHE, lucigenin and pharmacological inhibitors to better evaluate O_2^- production. In the present study we have not used aromatase or 5-alpha reductase inhibitors to determine whether estradiol or dihydrotestosterone contribute to testosterone effects. However, in previous studies, testosterone effects were not blocked or modified by anastrozole (aromatase inhibitor). Finally, the time of treatment as well as the dose of testosterone were based on previous studies that investigated other metabolic and cardiovascular parameters in different experimental models.

CONCLUSION

Our findings provide evidence that supraphysiological levels of testosterone impairs vascular function via activation of the NLRP3 inflammasome in vascular cells. The generation of mitochondrial ROS is crucial for activation of the NLRP3 inflammasome. Pharmacologic inhibition or genetic deletion of the NLRP3 in mice protects from testosterone-induced vascular dysfunction. Our study highlights the importance of NLRP3 inflammasome in vascular dysfunction promoted by supraphysiological levels of testosterone.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use (CEUA) of the University of São Paulo, Ribeirao Preto, Brazil (Protocol n° 032/2018).

AUTHOR CONTRIBUTIONS

JA, RC, NL, and RT designed the study. JA, RC, CP, AF, and CS conducted the experiments. JA, RC, and RT provided supplies and analytical tools. JA, RC, CP, and AF performed the data analysis. JA, RC, FC, NL, and RT wrote the paper. All authors contributed to the article and approved the submitted version.

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Androgen Receptor Signaling Positively Regulates Monocytic Development

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Myeloid cells are critical cells involved in the orchestration of innate and adaptive immune responses. Most myeloid cells derive from the adult bone marrow in a process called myelopoiesis, a tightly controlled process that ensures constant production of myeloid cells. Sex differences in myeloid cell development have been observed; males exhibit greater monocytic differentiation in the bone marrow, and men have increased blood monocyte numbers when compared to women. Here we use a genetic mouse model of myeloid androgen receptor (AR) knockout (MARKO) and pharmacological inhibition of AR to investigate the role of androgen signaling in monocytic differentiation. We observe that although myeloid AR signaling does not influence total bone marrow cell numbers, it does affect the composition of the bone marrow myeloid population in both homeostatic and emergency settings. Genetic deletion of AR in myeloid cells led to reduced monocytic development *in vivo*. Similarly, pharmacologic inhibition of AR signaling *in vitro* reduced monocytic development. However, alteration in monocytic differentiation in the absence of AR signaling did not lead to reduced numbers of circulating myeloid cells, although MARKO male mice display reduced ratio of classical to non-classical monocytes in the blood, implying that blood monocyte subsets are skewed upon myeloid AR deletion. Our results suggest that the sex differences observed in monocytic differentiation are partly attributed to the positive role of the androgen–AR axis in regulating monocytic development directly at the myeloid cell level. Furthermore, we have identified a novel role for AR in regulating blood mature monocyte subset turnover. Investigating how androgen signaling affects monocytic development and monocyte subset heterogeneity will advance our understanding of sex differences in monocytic function at homeostasis and disease and can ultimately impact future therapeutic design targeting monocytes in the clinic.

Keywords: androgen receptor, enzalutamide, monocyte development, myelopoiesis, 5-FU (5-fluorouracil)

INTRODUCTION

Myeloid cells are critical to tissue homeostasis and in the orchestration of innate and adaptive immune responses. Monocytes are a heterogeneous set of myeloid cells composed of different sizes and functions. Monocytes have been implicated in diseases such as atherosclerosis and cancer (1). Understanding the regulation of monocytic production in homeostatic and stress settings can ultimately lead to the development of therapies that modulate monocyte numbers and function in disease. Myeloid cell development, or myelopoiesis, is a tightly regulated process that occurs in the adult bone marrow (BM), to ensure constant production of myeloid cells throughout an individual's life (2, 3). During monocytic development, monocytic growth factors, such as granulocyte-macrophage and macrophage colony-stimulating factors (GM- and M-CSF respectively), and specific transcription factors coordinate the development of myeloid progenitors in specific BM niches. The earliest myeloid progenitors, known as common myeloid progenitors (CMPs), give rise to granulocyte/monocyte progenitors (GMPs); and within the GMP population, a monocytic progenitor (MoP) subpopulation produces mature monocytes that exit the BM and circulate in the bloodstream (4, 5). Monocytes that enter the blood circulation are named classical monocytes (CMs). Most of CMs leave the circulation to replenish myeloid subpopulations in different organs, while a few remain in the blood and transition into intermediate monocytes (IMs), and subsequently into non-classical monocytes (NCMs) (6). Blood NCMs patrol vasculature to remove damaged cells or debris (6). At inflammatory sites, such as in the tumor microenvironment or infection sites, CMs differentiate into monocyte-derived macrophages or dendritic cells depending upon microenvironmental cues. A constant production of monocytes and the ability to upregulate monocytic cells upon stress are key and impact inflammation, disease progression, and resolution (7).

Sex differences have been observed in disease prevalence and outcome (8). Interestingly, sex differences in monocyte development have also been described and may be a mechanism involved in sex-biased disease progression. M-CSF is expressed at higher levels in the BM of male mice compared to female mice (9). In addition, male BM cells cultured in colony-forming assays show increased monocyte and monocyte–granulocyte colony-forming units (CFU-M and CFU-GM) numbers when compared to females, indicating increased monocytic differentiation (9). Replenishment of peritoneal macrophages from BM monocytes is increased in male *versus* female mice (10, 11). During inflammatory settings, such as obesity, male mice have increased proportions of myeloid cell progenitors when compared to females (12). During inflammation of the peritoneum, male mice show greater increases in blood classical monocytes when compared to females (13). In humans, men have higher proportion of blood monocytes when compared to women (14). The sex bias in monocytic development occurs in post-pubertal setting where fluctuations in sex hormones occur, suggesting a role for sex hormone signaling in the regulation of monocytic development. Specifically, a positive role for testosterone and androgen receptor (AR) signaling may explain the sex differences observed. Studies examining the effects of AR signaling in myeloid cell development provide initial hints into potential mechanisms. Global AR knockout (GARKO) mice display no changes in CMP progenitors (15), although there is an observed decrease in bone marrow macrophages in GARKO relative to WT mice (16). Moreover, G-, GM- or M-CSF treatment of GARKO or WT bone marrow cells does not induce changes in CFU-G, -GM or -M, suggesting AR expression in progenitors is not involved with GMP differentiation (15). Nonetheless, GARKO mice are neutropenic and show reduced percentage of monocytic cells in the blood as compared to WT mice (15, 16). However, it remains unclear how AR expression in either progenitors or mature myeloid cells affects myelopoiesis in homeostasis and under stress conditions.

In this study, we utilized a genetic mouse model in which AR expression in myeloid cells has been genetically deleted (MARKO) and pharmacological inhibition of AR to understand the role of AR on myeloid cell development under homeostasis and myeloablative stress. We show that AR positively regulates BM monocytic development and that myeloid cell AR deletion alters the BM myeloid compartment. We further identify that AR expression in myeloid cells impacts blood monocyte subsets, with MARKO male mice exhibiting decreased percentage of CM. Further, the rate of monocytic production is impaired in MARKO male mice under myeloablative conditions. Importantly, we identify that AR positively affects monocytic differentiation by impacting not only mature myeloid cells, but also by reducing monocytic differentiation at the progenitor level.

MATERIAL AND METHODS

Animal Studies

Seven to ten week-old C57BL/6 male mice were purchased from Taconic Laboratory (Hudson, NY); $LysM^{cre}$ C57BL/6 male and B6.129(Cg)-Gt(ROSA)26$Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). AR^{floxed} mice were generated by De Gendt Lab at Katholieke Universiteit Leuven (17) and kindly shared by Agoulnik Lab at Florida International University. For the generation of Myeloid AR KnockOut (MARKO) mice, Lys-M^{cre} males were crossed with AR^{floxed} females to generate MARKO males. Control male mice for the experiment, referred to as WT mice, consisted of either AR^{floxed} littermates or syngeneic C57BL/6 male mice. Mice were housed in microisolator cages in a laminar flow unit under ambient light at 24°C. The RPCCC Institutional Animal Care and Use Committee (IACUC) approved all procedures and experiments for this study.

In Vivo Experiments

For *in vivo* enzalutamide treatments, WT C57BL/6 male mice were treated daily with 100 µl of 20 mg/kg enzalutamide (Selleckchem S1250) by oral gavage or vehicle for 5 or 14 days.

For emergency myelopoiesis experiments, WT and MARKO male mice were treated i.p. with 150 mg/kg 5-fluorouracil (5-FU, Invivogen sud-5fu) intraperitoneally and followed over time.

Blood Analyses

Terminal blood collection was performed following CO_2 euthanization of mice. Blood leukocyte populations were determined by complete blood count (CBC) analysis. For flow cytometric analyses of blood leukocytes, blood was collected, RBC lysed and stained with surface antibodies (below).

Primary Cultures

Total bone marrow from C57BL/6 mice was initially lineage depleted by incubation with anti-mouse lineage antibodies (Anti-B220 eBioscience cat# 14-0452-82, anti-CD11b eBioscience cat# 14-0112-82, anti-Ter119 eBioscience cat# 14-5921-82) using magnetic beads (Qiagen cat# 310107). Lineage depleted bone marrow was then FACS-sorted for oligopotent GMP cells based on expression of specific markers (Sca-1$^-$ c-Kit$^+$ CD16/32$^+$ CD150$^-$ Ly6C$^-$ CSF1R$^-$). Sorted oligopotent GMPs were cultured in 10 ng/ml of IL-3 (Preprotech AF-213-13) and 10 ng/ml of SCF (Preprotech AF-250-03) in 200 µl of phenol red free RPMI 1640 (ThermoFisher 11835030) supplemented with 100 µg/ml Penicillin–Streptomycin–Glutamine (ThermoFisher 10378016) and 10% FBS Premium Select (Atlanta S11595) at 37°C and 5% CO_2. Experiments were performed in the presence/absence of 5 µM enzalutamide (Selleckchem S1250) in 96 well plates at 1,000 cells/well. The concentration of enzalutamide utilized was determined previously (18), and was chosen based on concentrations used in the literature for *in vitro* cultures (19, 20). Experiments with M-CSF stimulation of oligopotent GMPs were performed in the same conditions in the presence of 10 ng/ml of M-CSF.

Bone marrow-derived macrophages (BMDMs) were generated by culturing 1×10^6 unfractioned bone marrow cells from C57BL/6 male mice with 30 ng/ml M-CSF (ThermoFisher 14-8983-80) in the presence of DMSO or 5 µM enzalutamide (Selleckchem S1250) in 10 cm dishes in phenol red free RPMI 1640 (ThermoFisher 11835030) supplemented with 100 µg/ml Penicillin–Streptomycin–Glutamine (ThermoFisher 10378016) and 10% FBS Premium Select (Atlanta S11595) at 37°C and 5% CO_2 for 5 days.

Flow Cytometry

For flow cytometry staining, single cell suspensions were incubated with antibodies against cell surface molecules for 40 min on ice. Cells were then washed twice, and analysis was done at flow cytometer (BD Fortessa, BD LSRII). Analyses were performed using FlowJo™. Using FlowJo plugins (https://flowjo.com/exchange/#/), dimensionality reduction was performed by downsizing samples to 25 K cells each, concatenating samples and performing UMAP using the default settings (Euclidean distance, nearest neighbors of 15, and minimum distance of 0.5). Percentage of cre-expressing myeloid cells was determined by flow cytometry using LysM^{cre} ROSA$^{mT/mG}$ mice. Antibodies used for flow cytometry were CD45 (BD 550994), CD11b (BD 553311), F4/80 (BioLegend 123149), CD115 (ThermoFisher 12-1152-82), Ly-6G (BioLegend 127612), Ly-6C (BioLegend 128033), CD16/32 (eBioscience 45-0161-82), Sca-1 (eBioscience 25-5981-82), CD150 (BioLegend 115927), c-Kit (BioLegend 105826), Ter-119 (eBioscience 48-5921-82), B220 (eBioscience 57-0452-82), CD4 (BioLegend 100427), CD8a (eBioscience 48-0081-82), CD3e (ThermoFisher MA5-17658), IRF8 (eBioscience 17-9852-82), PU.1 (Cell signaling 2216S), CD43 (BD 560663), and LD UV (ThermoFisher L23105).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 software. When comparing two groups, statistical analyses were performed using two-tailed Mann–Whitney or paired tests. When comparing two groups or more groups, 1-way or 2-way ANOVA was performed. Multiple comparison correction (Bonferroni correction) was applied when necessary. Differences were considered significant when P values were ≤ 0.05.

RESULTS

Androgen Receptor Is Implicated in Bone Marrow Monocytic Development

Sex differences are observed in monocyte development in the bone marrow and blood monocyte levels; males generally have increased levels of monocytes (9–14). To understand whether testosterone activation of AR signaling accounts for this sex difference and affects monocytic cell development in male mice, we generated Myeloid AR KnockOut (MARKO) mice, which lack AR expression in mature myeloid cells (**Supplementary Figure 1**). Bone marrow of WT and MARKO male mice was analyzed by flow cytometry using dimensionality reduction of single cells by uniform manifold approximation and projection (UMAP) algorithms (21). UMAP was performed on singlet live lineage negative cells (Lineage CD3, Ly-6G, B220, Ter119) to visualize clusters of developing myeloid cells and mature non-granulocytic myeloid cells in an unbiased manner. Cell clusters were identified using the parameters c-Kit, Sca-1, CD150, CD16/32, Ly-6C, CD115, CD11b and F4/80, with WT and MARKO male BM displaying differences in clusters (**Figure 1A**). To define the different populations, we analyzed the expression of myeloid markers within the UMAP coordinates. We identified differences between WT and MARKO BM, with MARKO BM displaying reduced CD115 expression within two populations, and increased expression of CD11b and F4/80 (**Figure 1B**, arrows indicate differences). To further dissect how AR expression impacts myeloid cell development, we analyzed developing myeloid cell populations by manual flow cytometry gating (**Supplementary Figure 2A**). WT and MARKO males did not display differences in total bone marrow cell numbers, nor in the percentage of granulocyte–monocyte progenitors (GMPs) (**Supplementary Figures 2B, C**). Further analysis of GMPs identified three subpopulations of GMPs: oligopotent GMP (oGMP), granulocytic progenitor (GP), and monocytic progenitor (MoP) populations (**Supplementary Figure 2D**). While AR deletion in myeloid cells did not affect oGMP and GP cell percentage and numbers (**Figure 1C** and **Supplementary Figure 2E**), MARKO male mice displayed significantly reduced MoP percentage and cell numbers (**Figure 1D**), suggesting a

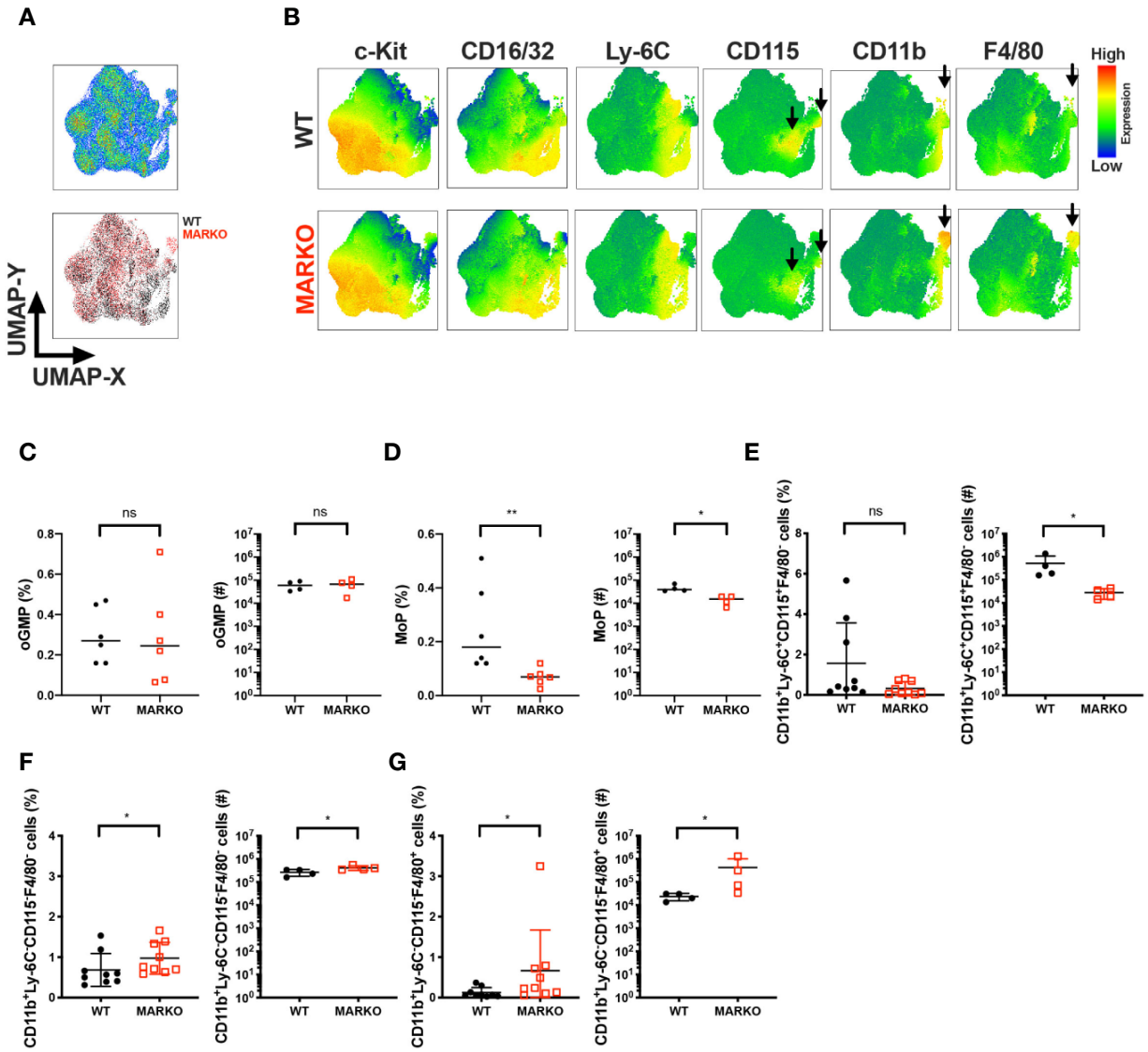

FIGURE 1 | AR is important for bone marrow monocytic development. BM of WT and MARKO male mice was analyzed by flow cytometry for monocytic and macrophage cell populations. **(A)** The upper plot represents UMAP analysis of live singlet lineage negative (CD3, Ly-6G, B220, Ter119) BM cells from WT and MARKO male mice. The lower plot in **(A)** is colored according to sample group. **(B)** UMAP graphs indicating intensities of c-Kit, CD16/32, Ly-6C, CD115, CD11b and F4/80 expression in WT and MARKO BM. **(C–G)** Quantification of BM cell populations utilizing gating schemes in **Supplementary Figures 1A, D, F**. Plots depict percentage and numbers of singlet live **(C)** oligopotent GMP (oGMP), **(D)** monocytic progenitors (MoPs), **(E)** $CD11b^+Ly6C^+CD115^+F4/80^-$ cells, **(F)** $CD11b^+Ly6C^-CD115^-F4/80^-$ cells, and **(G)** $CD11b^+Ly6C^-CD115^-F4/80^+$ cells. Graphs show pooled data from two to four experiments with two to three mice per group. Black filled squares denote WT, and red empty squares indicate MARKO BM. Comparisons were done with non-parametric *t*-test. ns, not significant; *p ≤ 0.05, **p ≤ 0.01.

positive role for AR during monocytic development. To further understand whether AR impacts the mature non-granulocytic myeloid compartment in the BM, mature myeloid cell populations were identified by manual flow cytometry gating (**Supplementary Figure 2F**). WT and MARKO BM did not differ in mature $CD11b^+Ly\text{-}6C^-CD115^+F4/80^-$, $CD11b^+Ly\text{-}6C^+CD115^-F4/80^-$, or $CD11b^+Ly\text{-}6C^-CD115^+F4/80^+$ myeloid cell populations (**Supplementary Figures 2G–I**). However, BM isolated from MARKO male mice displayed reduced numbers of mature $CD11b^+Ly\text{-}6C^+CD115^+$ BM monocytes when compared to BM from WT mice (**Figure 1E**). Moreover, MARKO BM displayed increased percentage and number of cells in the non-monocytic and non-granulocytic $CD11b^+Ly\text{-}6C^-CD115^-F4/80^-$ population (**Figure 1F**) and increased percentage and number of cells in the macrophage $CD11b^+Ly\text{-}6C^-CD115^-F4/80^+$ population (**Figure 1G**) when compared to WT BM, corroborating the differences visualized through UMAP (**Figure 1B**). These results suggest that AR expression significantly impacts the myeloid bone marrow compartment by increasing monocytic development.

Androgen Receptor Impacts the Percentage of Blood Monocyte Subset

It is possible that the reduction in BM monocytes observed in MARKO mice impacts circulating blood monocyte numbers. To test this hypothesis, we analyzed blood leukocyte numbers of WT and MARKO male mice by complete blood counts (CBC). No differences were observed in total white blood cell (WBC) numbers (**Supplementary Figure 3A**), nor in the individual myeloid populations of neutrophils, monocytes, eosinophils, and basophils (**Figure 2A**); thus, AR deletion does not appear to affect blood monocyte numbers. To understand whether AR alters monocyte subsets, we quantified the percentage of $CD11b^{+}CD115^{+}$ monocyte subsets within leukocytes in WT and MARKO male blood by flow cytometry. Classical (CM), intermediate (IM), and non-classical (NCM) monocyte subsets were identified based on Ly-6C and CD43 expression (**Figure 2B**). Indeed, MARKO males exhibited altered percentage of monocyte subsets when compared to WT males, with a decreased percentage of CM and reduced ratio of the percentage of CM to NCM (**Figures 2C–D**). These results imply that AR positively regulates BM monocytic development and blood CM subset.

The Rate of Monocytic Production Is Impaired in MARKO Male Mice

To understand how myeloid AR affects the rate of monocytic production, mice were exposed to myeloablative therapy by intraperitoneal injection with a single sublethal dose of 5 fluorouracil (5-FU). Recovery of BM and blood cells was analyzed 8, 10, 12, and 14 days after 5-FU injection (**Figure 3A**). Myeloablative therapy induced weight loss in both WT and MARKO mice; MARKO males showed small but significant increase in weight 14 days after 5-FU injection when compared to WT males (**Supplementary Figure 3B**). No differences in recovery of total BM cell count were observed (**Supplementary Figure 3C**). GMP production peaked at day 10 following 5-FU for both WT and MARKO mice, and percentage of GMPs were similar between WT and MARKO mice (**Supplementary Figure 3D**). BM isolated from MARKO mice displayed significantly increased percentage of oGMP, and no changes in the percentage

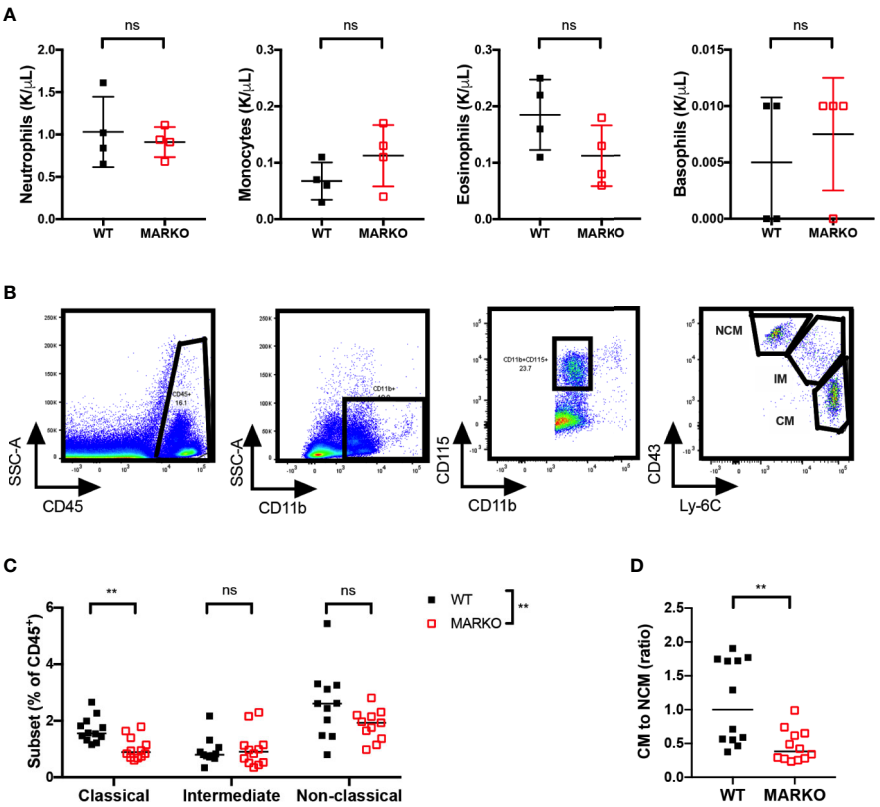

FIGURE 2 | AR deletion decreases ratio of classical to non-classical blood monocytes. Blood leukocytes of WT and MARKO male mice were collected and assessed by complete blood count (CBC) and flow cytometry. Plots depict quantification of blood **(A)** neutrophils, monocytes, eosinophils, and basophils by CBC. **(B)** Flow cytometry gating strategy utilized to distinguish classical (CM), intermediate (IM), and non-classical (NCM) blood monocytes. **(C)** Monocyte subset percentage out of total $CD45^{+}$ blood cells. **(D)** Ratio of the percentage of blood CM to NCM. Graphs show pooled data from two to four experiments with two to three mice per group. Black filled squares denote WT, and red empty squares indicate MARKO BM. Graph **(C)** was compared by two-way ANOVA, comparisons in **(A, D)** were done with non-parametric *t*-tests. ns, not significant; $^{**}p \leq 0.01$.

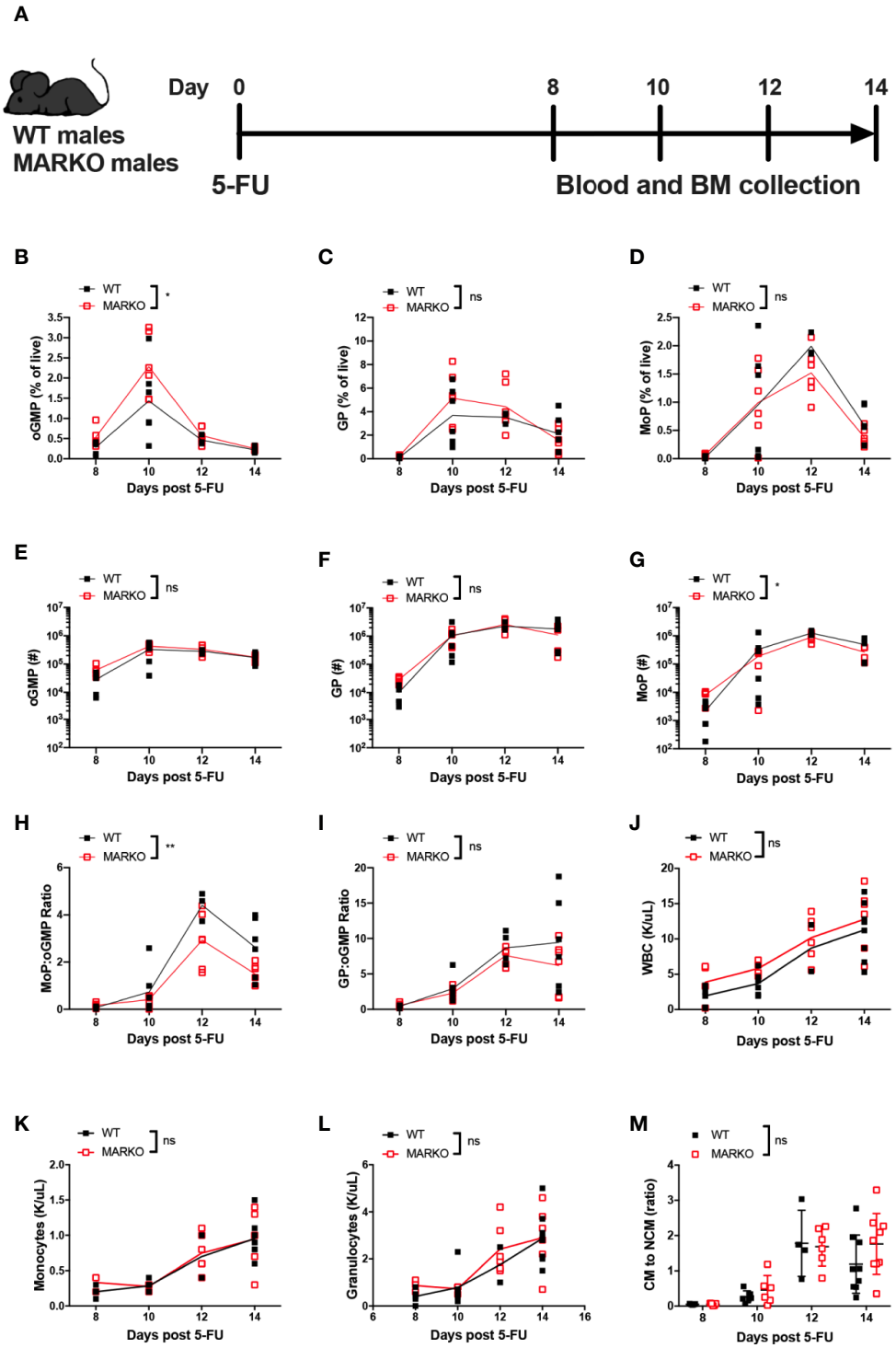

FIGURE 3 | Deletion of myeloid cell AR is associated with reduced bone marrow monocytic progenitor production following 5-FU bone marrow ablation. **(A)** Experimental design: WT and MARKO male mice were injected intraperitoneally with 5-fluorouracil (5-FU), and BM and blood samples were collected 8, 10, 12 and 14 days following treatment. Plots depict percentage of singlet live BM **(B)** oligopotent GMPs (oGMPs), **(C)** granulocytic progenitors (GPs), and **(D)** monocytic progenitors (MoPs). Graphs indicate total number of BM **(E)** oGMP, **(F)** GP, and **(G)** MoP. Graphs denote ratio of the percentage of BM subpopulation **(H)** MoP to oGMP, and **(I)** GP to oGMP. **(J–L)** CBC quantification of **(J)** total white blood cells (WBCs), **(K)** blood monocytes, and **(L)** blood granulocytes. **(M)** Ratio of blood classical monocytes (CM) to non-classical monocytes (NCMs). Graph shows data from two to three experiments with two mice/group/time point. Black filled squares denote WT, and red empty squares indicate MARKO samples. Statistical analyses were done by two-way ANOVA. ns, not significant; *$p \leq 0.05$, **$p \leq 0.01$.

of GP and MoP were produced; however, MARKO BM MoP numbers showed a significant reduction following 5-FU when compared to WT mice (**Figures 3B–G**). These results suggest that rather than faster production and early release of monocytes into the bloodstream, MARKO mice fail to produce as many monocytic progenitors as WT mice following myeloablative therapy. Indeed, the ratio of MoP to oGMP production, but not the ratio of the percentage of GP to oGMP, is reduced in MARKO mice when compared to WT mice, indicating a specific impairment in the monocytic differentiation pathway (**Figures 3H, I**). Nonetheless, the reduced rate of monocytic production in MARKO does not affect recovery of blood WBC, granulocyte or monocyte numbers following 5-FU (**Figures 3J–L**). No differences were observed in the percentage of blood CM, IM, and NCM, nor in the ratio of CM to NCM during the 14 days of recovery (**Figure 3M** and **Supplementary Figures 3E–G**).

Pharmacological AR Antagonism Reduces Monocytic Differentiation at the Progenitor Level

To identify which cell types contributed to the changes in monocytic development observed in MARKO males, blood and BM from $LysM^{cre}$ $ROSA^{mT/mG}$ mice were collected, and percentage of cre-expressing myeloid cells was analyzed by flow cytometry to evaluate MARKO model penetrance of AR deletion during myelopoiesis. AR deletion was observed in about half of BM macrophages, identified as bulk $CD11b^{+}F4/80^{+}$ cells. (**Figure 4A**). Further, BM progenitor populations of oGMP, GP and MoP had very low penetrance of AR deletion (~5–10% of progenitor populations), while 50–60% of blood monocyte subsets lacked AR (**Figure 4A**). These results suggest that the effects observed in MARKO male mice occur as monocytes are differentiating, and it is likely that mature BM myeloid cells are influencing the monocytic differentiation, as has been previously demonstrated (22–24). To test the hypothesis that AR signaling can also impact monocytic differentiation at the progenitor level, BM oGMP cells were sorted from WT males, cultured in media containing stem cell factors IL-3 and SCF in the presence or absence of the second-generation AR antagonist enzalutamide; differentiation was assessed after 3 days of culture by flow cytometry (**Figure 4B** and **Supplementary Figures 4A–B**). Differentiation of oGMP was quantified based on c-Kit, CD115, and Ly-6C expression (**Figure 4C**). After 3 days of culture, the majority of cells in culture differentiated in both groups, as the percentage of $c\text{-}Kit^{-}$ cells was higher than $c\text{-}Kit^{+}$ (**Figures 4D, E**). AR antagonism resulted in delayed differentiation, as the percentage of progenitor cells (oGMP and MoP) was significantly higher in enzalutamide-treated when compared to untreated samples (**Figure 4D**). Enzalutamide skewed the differentiation of oGMPs towards non-monocytic $c\text{-}Kit^{-}$ cells, as the percentage of $Ly\text{-}6C^{-}CD115^{-}$ cells increased while the percentage of both $Ly\text{-}6C^{+}CD115^{-}$ and $Ly\text{-}6C^{+}$ $CD115^{+}$ cells decreased in enzalutamide-treated when compared to untreated cultures (**Figure 4E**). These results suggest that in the absence of a monocytic differentiating factor, AR inhibition blocks monocytic differentiation at the progenitor level and results in increased non-monocytic differentiation.

Monocytic differentiation in the bone marrow is transcriptionally regulated and requires coordination of specific monocytic growth factors, such as GM-CSF, M-CSF and IL-34 (25–27). To test whether AR antagonism impacted monocytic differentiation in the presence of a monocytic growth factor, unfractioned WT BM cells were cultured *in vitro* with M-CSF in the presence of enzalutamide or DMSO for 5 days, and viable cell numbers were assessed. AR antagonism significantly reduced the number of monocyte/macrophage cells over 5 days of culture (**Figure 5A**). To further delineate the effect of AR antagonism on progenitors in the presence of a differentiating signal, BM oGMPs were sorted from WT males, cultured in media containing IL-3, SCF, and M-CSF in the presence or absence of enzalutamide, and differentiation was assessed after 1 and 3 days of culture by flow cytometry (**Figure 5B**; sorting strategy in **Supplementary Figures 4A, B**, and gating strategy for cultured cells in **Figure 4C**). oGMP cells cultured in the presence of M-CSF displayed increased differentiation over time, with the percentage of $c\text{-}Kit^{+}$ cells decreasing in both groups from days 1 to 3 of culture (**Figure 5C**). Enzalutamide treatment did not result in differences from untreated samples in the percentage of progenitor populations over time. However, the percentage of differentiated non-monocytic $Ly\text{-}6C^{-}CD115^{-}$ cells significantly increased over time in enzalutamide-treated samples when compared to untreated even in the presence of a monocytic differentiating signal (**Figure 5C**). Enzalutamide treatment also reduced percentage of differentiated $Ly\text{-}6C^{+}CD115^{-}$ cells over time when compared to untreated (**Figure 5C**). In addition, the upregulation of the M-CSF receptor CD115 and the mature myeloid and macrophage markers CD11b and F4/80 were significantly reduced over time upon AR blockade as compared to control cultures (**Supplementary Figure 4C**). To visualize the effect of AR antagonism in monocytic differentiation *in vitro*, UMAP plots were generated utilizing singlet $CD16/32^{+}$ cells after 3 days of culture in the presence of M-CSF. Unsupervised analysis revealed differential proportion of clusters between untreated and enzalutamide-treated cultures (**Figure 5D**). Enzalutamide-treated cultures displayed clusters with reduced CD115, CD11b, F4/80, and Ly-6C expression and no changes in c-Kit and CD16/32 (**Figure 5E** and **Supplementary Figure 4D**). Altogether these results suggest that AR antagonism skews progenitor differentiation away from monocytic development even in the presence of a monocytic differentiating signal.

DISCUSSION

Understanding how sex hormone receptors impact myelopoiesis is key to dissecting mechanisms involved in monocytic function in health and disease. Here we demonstrate that AR blockade at both the progenitor and mature myeloid cell level leads to changes in BM composition, and results in reduced monocytic differentiation in male mice (**Figure 6**).

Using a murine model with AR-deficient myeloid cells, we observe a reduction in different $CD115^{+}$ monocytic cell populations, from monocytic progenitors to mature BM

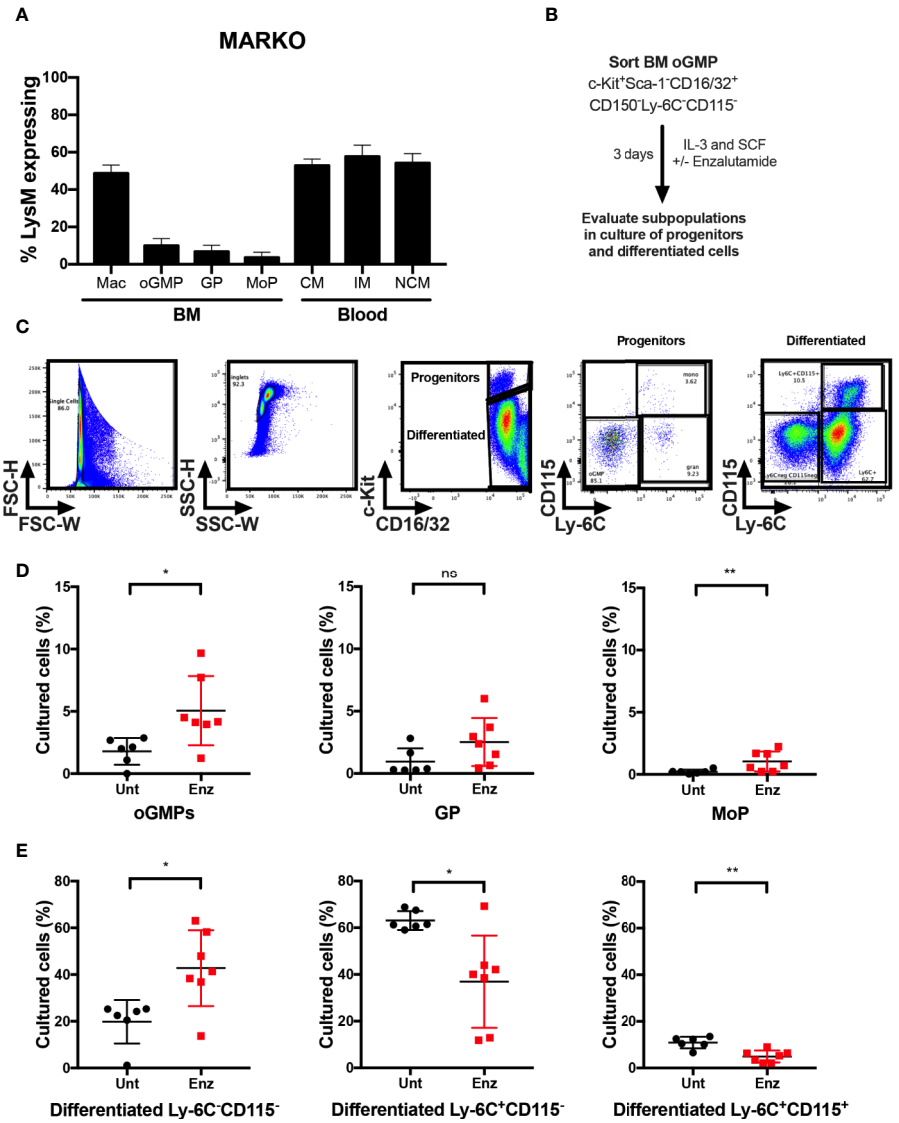

FIGURE 4 | AR antagonism delays monocytic development *in vitro* in the absence of differentiating signals. **(A)** Bone marrow and blood of LysM^{cre} ROSA$^{mT/mG}$ mice were collected and percentage of cre-expressing myeloid cells was analyzed by flow cytometry to evaluate MARKO model penetrance. **(B)** Experimental design: Oligopotent GMP (oGMP) population was sorted from WT male BM and cultured *in vitro* with stem cell factors IL-3 and SCF in the presence or absence of enzalutamide for three days and differentiation was assessed by flow cytometry. **(C)** Flow cytometry gating of untreated and enzalutamide-treated cultured cells on day 3. **(D, E)** Graphs depict percentage of **(D)** progenitor oGMP, granulocytic progenitors (GPs), monocytic progenitors (MoPs) and **(E)** differentiated cell populations in cultures at day 3. Graphs show data of three experiments with two biological replicates each. Black filled squares denote WT untreated, and red filled squares indicate enzalutamide-treated samples. Comparisons were done by non-parametric *t*-test. ns, not significant; *p ≤ 0.05, **p ≤ 0.01.

monocytes. Reduced ratio of MoP to oGMP but no increases in GP to oGMP ratio following myeloablation with 5-FU suggests that AR deletion can block monocytic differentiation rather than skew GMPs towards granulocytic differentiation. Similarly, oGMP differentiation into MoP was diminished in the presence of a pharmacological AR inhibitor *in vitro* when M-CSF was both present and absent. Our results imply that AR is important for monocytic differentiation, and lack of AR signaling reduces monocytic differentiation. In addition, enzalutamide treatment decreases monocyte/macrophage cell numbers when bulk bone marrow containing HSCs is cultured with M-CSF. These results suggest that not only differentiation, but also proliferation and/or survival is impacted by AR expression in myeloid cells. The reduced numbers of cultured cells could be a consequence of increased cell death or slower proliferation. In prostate cells, AR expression is associated with cell survival and proliferation. In prostate cancer, androgens can stimulate cell cycle progression by AR-mediated regulation of G1–S transition,

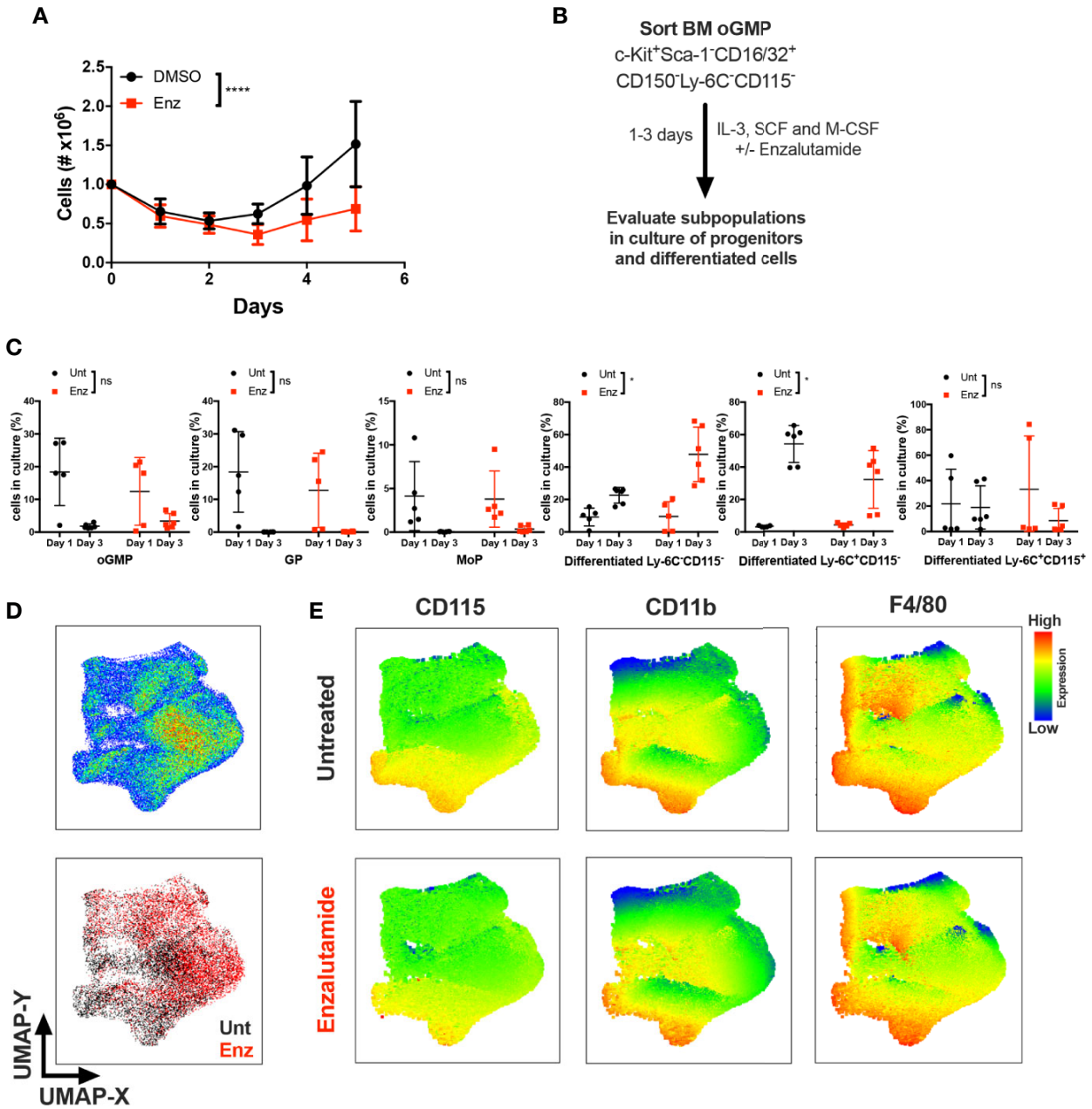

FIGURE 5 | AR antagonism delays monocytic development *in vitro* in the presence of differentiating M-CSF signal. **(A)** Unfractioned WT bone marrow cells were cultured *in vitro* with M-CSF in the presence of DMSO or enzalutamide (Enz) for 5 days. Graph depicts **(A)** cell numbers after 1, 2, 3, 4, and 5 days of culture. **(B)** Experimental design, where oligopotent GMP (oGMP) population was sorted from WT male BM, cultured *in vitro* with IL-3, SCF, and M-CSF, and left untreated (Unt) or treated with enzalutamide for one and three days. Differentiation was assessed by flow cytometry. **(C)** Using gating strategy from **Figure 4C**, graphs depict percentage of oGMP, granulocytic progenitors (GPs), monocytic progenitors (MoPs) and differentiated cell populations in cultures at days 1 and 3. **(D)** The upper plot represents UMAP analysis of cultures at day 3 gated at singlet CD16/32$^+$ population. The lower plot in **(D)** is colored according to sample group. **(E)** UMAP graphs indicate intensities of CD115, CD11b, and F4/80 expression in untreated and enzalutamide-treated cultures at day 3. Graphs show data of three experiments with one to two biological replicates/group each. Black filled squares denote WT untreated, and red filled squares indicate enzalutamide-treated samples. Statistical analyses in **(A, C)** were done by two-way ANOVA. ns, not significant; *p ≤ 0.05, ****p ≤ 0.0001.

while androgen ablation triggers cell death and cell cycle arrest (28). Conversely, AR knockout in B cells is associated with increased proliferation and decreased apoptosis of these cells (29), while AR knockout in neutrophils reduces the proliferative capacity of neutrophils and retards their maturation (15). Our findings relate to the previous study, where we observe delayed monocytic maturation in MARKO males. Therefore, it is possible that AR might be involved not only in differentiation,

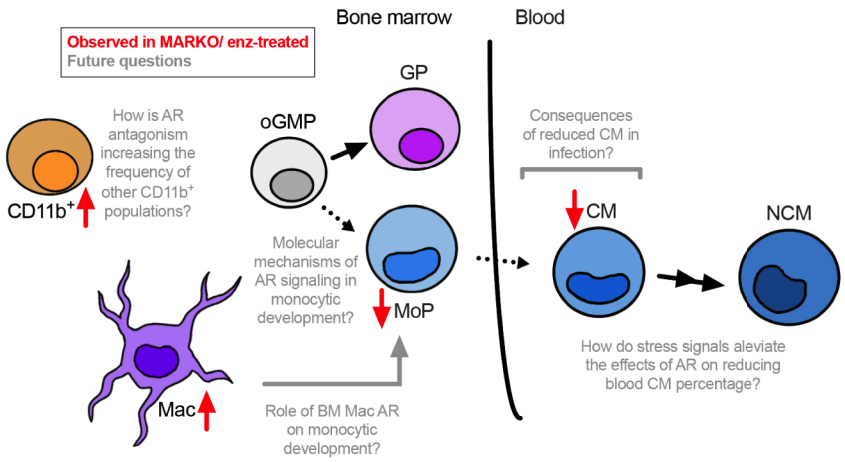

FIGURE 6 | Overall model. AR positively regulates monocytic development. Through *in vitro* studies using enzalutamide (enz) and *in vivo* experiments using myeloid AR knockout (MARKO) male mice, we demonstrate that lack of AR is involved with reduced monocytic development in the bone marrow and increased presence of mature BM cells, such as macrophages (Mac) and non-granulocytic non-monocytic CD11b$^+$ cells. In addition, reduced monocytic development is accompanied by changes in blood monocyte subsets, where myeloid AR deletion reduced the percentage of classical monocytes (CMs). Several questions can now be pursued in terms of molecular mechanisms by which AR impacts monocytic development and its consequences during inflammatory or infectious conditions.

but also in the proliferative capacity of monocytic progenitors. However, AR does not appear to regulate recovery following 5FU-induced emergency myelopoiesis.

As monocytic differentiation was reduced in MARKO males under homeostatic and stress conditions, we hypothesized that this would result in reduced blood monocyte levels. Yet, MARKO male mice displayed normal monocyte blood counts; the lower classical to non-classical blood monocyte ratio in MARKO male mice implies that AR signaling affects developing monocytes as well as mature monocyte subsets. Nonetheless, monocyte subset ratio is not affected up to 14 days following 5-FU. These results suggest stress signals may induce compensatory mechanisms that alleviate effects of AR on blood monocytes under stress conditions. Alternatively, the change in monocytic subsets in homeostasis can imply differential monocytic subset lifespan, migration or differentiation. Recently, we have shown that myeloid AR does not affect myeloid cell infiltration into subcutaneously implanted C2 prostate tumors, suggesting that AR does not affect blood monocytes tumor migration (18). Alveolar macrophages from MARKO male mice display decreased cytokine and chemokine production in a model of allergic lung inflammation, and these changes are associated with decreased eosinophil infiltration to lung (30). It still remains to be determined how AR is impacting monocyte subsets in models of infection and inflammation, and how decreased CM to NCM ratio impacts disease susceptibility and progression. As men have higher proportion of blood monocytes when compared to women, and monocyte proportions increase in women with age (14, 31), future studies should compare blood monocytes between sexes across lifespan to determine how sex hormone variation impacts these subsets.

We show that MARKO male mice have higher BM non-granulocytic non-monocytic CD11b$^+$ cells and BM macrophages concomitantly with the reduction in BM monocytic differentiation. These results bring about two potential hypotheses: AR deletion skews differentiation of progenitor cells towards other non-monocytic myeloid cell types, and/or AR differentially impacts the survival of different myeloid population(s). Our *in vitro* experiments indicate that direct AR antagonism of GMPs leads to increased percentages of non-monocytic non-granulocytic CD11b$^+$ cell in cultures even in the presence of M-CSF, indicating that AR acts directly on progenitors to skew their differentiation. More studies will be needed to specifically assess how GMP differentiation is being altered and which lineages are increasing with enzalutamide treatment, such as DC, basophil, and eosinophil lineages. Thus, it remains to be determined how AR expression in mature myeloid cells affects monocytic cell numbers in the BM. AR expression in mature myeloid cells may affect the bone marrow microenvironment to alter monocytic differentiation. M-CSF is expressed at higher levels in male BM compared to female (9), and it is possible that AR positively regulates M-CSF expression. Another factor involved in the activation of CSF-1R is IL-34, which has not been assessed in this study and could be involved with the reduced monocytic number observed. In addition, BM macrophages have been shown to maintain HSC niches (22, 23), while bone marrow myeloid cells are closely associated with developing myeloid cells and affect their quiescence and self-renewal (24). It is therefore possible that AR deletion in mature myeloid cells impacts monocytic development by disrupting/altering the communication between mature and developing myeloid cells.

By using a myeloid-specific knockout model, we were able to determine the specific contribution of myeloid AR to monocytic development. Some of our results contrast to previous work using a global AR knockout model, where decreased bone marrow macrophages and reduced percentage of blood monocytes were observed in GARKO mice when compared to WT (15, 32). This discrepancy could be a result of i) different penetrance of AR deletion in myeloid cells between the models,

and/or ii) additive or compensatory effects of AR deletion in non-myeloid cells of GARKO mice. As androgens affect B cell development through modulation of androgen-sensitive BM stromal cells (33), it is possible that stromal cell AR also impacts monocytic development. Future studies should address the effect of AR expression by different cell populations on monocytic development to tease out how individual contribution of each component impacts the overall effect.

Overall, our results indicate that AR is an important player in the process of monocytic development and may explain some sex differences observed in monocyte biology. Furthermore, our results imply that monocytic development may be affected by androgen levels and therapies that aim on reducing androgen signaling, such as AR antagonists used in prostate cancer treatment. Understanding how sex hormones and sex hormone receptors regulate monocytic production in homeostatic and stress settings may ultimately aid in the development of therapies that modulate monocyte numbers and function in disease.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Roswell Park Comprehensive Cancer Center (RPCCC) Institutional Animal Care and Use Committee (IACUC) approved all procedures and experiments for this study.

AUTHOR CONTRIBUTIONS

Conceptualization: CC and SG. Methodology: CC. Investigation and data analysis: CC and SG. Writing of original draft: CC and SG. Editing of original draft: CC and SG. Funding acquisition: SG. Supervision: SG. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.519383/full#supplementary-material

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