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Anti-inflammatory effects of androgens in the human vagina

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Abstract

Chronic inflammation is involved in the genitourinary syndrome of menopause (GSM) and beneficial effects of androgens in the vagina have been described. We investigated the potential involvement of human vagina smooth muscle cells (hvSMCs) in the inflammatory response and the immunomodulatory effect of androgen receptor (AR) agonist dihydrotestosterone (DHT). HvSMCs isolated from menopausal women were evaluated for sex steroids receptors and toll-like receptors mRNA expression, and left untreated or treated in vitro with lipopolysaccharide (LPS) or IFNγ, in the presence or absence of DHT. We evaluated mRNA expression (by RT-PCR) and secretion in cell culture supernatants (by a bead-based immunoassay) of pro-inflammatory markers. Nuclear translocation of NF-κB (by immunofluorescence) and cell surface HLA-DR expression (by flow cytometry) were also evaluated. Similar experiments were repeated in rat vSMCs (rvSMCs). In hvSMCs and rvSMCs, AR was highly expressed. DHT pre-treatment inhibited LPS-induced mRNA expression of several pro-inflammatory mediators (i.e. *COX2*, *IL-6*, *IL-12A* and *IFNγ*), effect significantly blunted by AR antagonist bicalutamide. DHT significantly counteracted the secretion of *IL-1RA*, *IL-2*, *IL-5*, *IL-15*, FGF, VEGF and TNFα. LPS-induced NF-κB nuclear translocation was significantly inhibited by DHT, an effect counteracted by bicalutamide. DHT pre-treatment significantly decreased IFNγ-induced expression of HLA-DR, mRNA expression of *iNOS, COX2* and *MCP1*, and secretion of IL-1, IL-2, IL-5, IL-6, MCP1 and GCSF. Similar effects were observed in rvSMCs. The activation of AR suppresses the inflammatory response in hvSMCs, reducing their potential to be involved in the initiation and maintaining of inflammation, thus representing a therapeutic strategy in conditions, such as the GSM.

Key Words

- androgens
- menopause
- vagina
- inflammation
- infection

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Introduction

For some women, menopause is an asymptomatic and unremarkable life transition, but for others it is accompanied by severe symptoms, such as hot flushes, mood changes and the so-called genitourinary syndrome of menopause (GSM), affecting approximately 50% of middle-aged and elderly women (Portman & Gass 2014). GSM construct encompasses a plethora of clinical manifestations, including vulvovaginal atrophy and lower urinary tract symptoms (LUTS) (Simon *et al.* 2018), which can impair quality of life by exerting detrimental effects on sexual health and general well-being (Simon *et al.* 2018).

The primary cause of GSM is a substantial and rapid drop of estrogen levels at menopause. However, a decline in androgen level is also likely to play a pathogenic role in the onset of GSM (Simon *et al.* 2018). Indeed, both changes in the genitourinary system and a progressive decline of circulating levels of total testosterone have been described in women throughout the aging process (Zumoff *et al.* 1980, Davison *et al.* 2005). Moreover, the androgen receptor (AR) is widely expressed in structures derived from the urogenital sinus, such as distal vagina, urethra and bladder, not only during embryogenesis (Cunha *et al.* 2017) but also throughout the adult life (Vignozzi *et al.* 2012*a*). Accordingly, several pre-clinical and clinical evidence indicates that androgens play a crucial role in the maintenance of genitourinary tissue structure and function in adulthood (Vignozzi *et al.* 2012*a*, Comeglio *et al.* 2016, Simon *et al.* 2018).

Adding layer to this complexity, chronic inflammation is a subtle underlying factor of GSM symptoms. The vaginal innate immune system is a unique and complex immunologic environment. Through an intricate balance among its major components, it is able to defend the genitourinary tract against pathogens, while maintaining immune-homeostasis and avoiding chronic inflammatory/autoimmune diseases. To accomplish this role, the human vagina contains several resident professional antigen presenting cells (APCs) (i.e. macrophages and dendritic cells) that recognize specific key molecules on the surface of pathogens, such as lipopolysaccharide (LPS), through the toll-like receptors (TLRs) system (Fazeli *et al.* 2005). Such recognition leads to pathogen clearance, leukocyte recruitment and antigen presentation to lymphocytes (Fazeli *et al.* 2005). Activation of TLRs is followed by a sustained release of proinflammatory cytokines and chemokines, including interleukin (IL) 1β (IL-1β), IL-6, tumor necrosis factor-α (TNFα), and chemokine (C-X-C motif) ligand 8 (CXCL8) (Pivarcsi *et al.* 2005). This acute response to infectious agents, to which the lower female genitourinary tract is particularly exposed, normally acts by removing the pathogens and initiating the healing process, leading to the restoration of tissue homeostasis. However, when resolution of the inflammation fails, chronic autoimmune inflammatory process may occur, as a result of uncontrolled differentiation and expansion of autoreactive CD4+ T helper (Th) cells, Th1 (Chen *et al.* 2017*b*).

Interestingly, in experimental models of chronic inflammatory diseases, androgens have shown prominent immunomodulatory and protective effects. In particular, in an experimental animal model of metabolic syndrome, testosterone treatment counteracted the chronic, multi-organ inflammation within the liver (Vignozzi *et al.* 2014), skeletal muscle (Sarchielli *et al.* 2020), and lower genitourinary tract tissues (Morelli *et al.* 2012, Vignozzi *et al.* 2012*b*). Noteworthy, in human prostatic stromal cells (Penna *et al.* 2009, Fibbi *et al.* 2010, Vignozzi *et al.* 2012*c*, 2013*a*,*b*, Comeglio *et al.* 2014), a well-validated non-professional APCs model, in vitro treatment with the selective AR agonist dihydrotestosterone (DHT) counteracted the expression and secretion of a plethora of cytokines and chemokines, induced by TNFα and LPS (Fibbi *et al.* 2010, Vignozzi *et al.* 2012*a*, 2013*a*,*b*). Although deep similarities in biological and molecular processes of male and female urogenital tracts have been demonstrated (Vignozzi *et al.* 2012*c*,*d*, Comeglio *et al.* 2016), the potential role of androgen-based therapies in the modulation of chronic genital inflammation in women has not been explored yet.

Present study is aimed at investigating, in human distal vagina smooth muscle cells (hvSMCs) isolated from post-menopausal women: (1) the expression of sex steroids receptors, in particular the AR, and of TLRs, which are usually expressed on resident APCs; (2) their potential involvement in the inflammatory response, acting as non-professional APCs and secreting a variety of proinflammatory mediators; (3) a possible anti-inflammatory effect of the selective AR agonist dihydrotestosterone (DHT). Similar experiments were also replicated in primary cell cultures derived from female rat distal vaginal tissues (rvSMCs) to evaluate possible species-specific differences. Our working hypothesis is that, similar to what previously demonstrated in the male genital tract (Vignozzi *et al.* 2012*a*), androgens might negatively modulate the inflammatory response in the vagina.

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Materials and methods

Surgical procedures and collection of biological samples

Human vagina and ovary tissues were obtained, after informed consent, from post-menopausal women undergoing surgery for benign gynecological disorders (study approved by the Ethics Committee 'Area Vasta Centro', Azienda Ospedaliero Universitaria Careggi, Florence, Italy: HUMVAGDHT, protocol no. 12903). Specifically, specimens from the upper third of the distal vagina, measuring approximately 3× 1 cm, were collected.

Sprague–Dawley female rats ($n=5$; Envigo, San Pietro al Natisone, Udine, Italy), weighting approximately 250 g, were individually caged under standard conditions in a temperature- and humidity-controlled room on a 12 h light:12 h darkness cycle, with free access to water and food throughout the study. After 8 weeks, the animals were killed by cervical dislocation, and the distal vagina and other tissues were collected for subsequent analysis. Animal handling complied with the Institutional Animal Care and Use Committee (IACUC) of the University of Florence, Italy, in accordance to the Italian Ministerial Law no. 26/2014.

Human and rat vagina smooth muscle cell cultures

Human smooth muscle cells (hvSMCs) were isolated from vaginal tissues and preliminary experiments were performed to validate the experimental protocol. Similarly, rat smooth muscle cells (rvSMCs) were isolated from distal vagina obtained from intact control female rats. The tissues biopsies were processed by mechanical and enzymatic dissection as previously described (Granchi *et al.* 2002, Comeglio *et al.* 2016). Briefly, single vagina tissue samples were digested with bacterial collagenase type IV (2 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ, USA) overnight at 37°C, the fragments were washed in PBS and centrifuged at 380 ***g*** for 10 min, the pellet was recovered and cultured in growth medium (GM) DMEM and Ham F-12 medium (DMEM/F12 1:1; Sigma-Aldrich) red phenol free supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (penicillin 100 IU/mL, streptomycin 100 mg/mL; Sigma-Aldrich), and amphotericin B (1 mg/mL; Sigma-Aldrich) in a fully humidified atmosphere of 95% air and 5% CO_2. SMCs began to emerge within 48 h and were used until the seventh/eighth passage.

For cytokines and chemokines mRNA expression analyses, hvSMCs and rvSMCs were seeded onto six-well plates (1.5×10^5 cells/well) in GM. After overnight serum starvation, the cells were left untreated (NT) or pre-treated with dihydrotestosterone (DHT, 30 nM; Sigma-Aldrich) with or without the non-steroidal anti-androgen bicalutamide (BICA, 1 μM; Sigma-Aldrich) for 24 h and then stimulated with LPS (100 ng/mL) or interferon γ (IFNγ 1000 UI/mL; R&D Systems) for 24 h. In preliminary experiments, the effect of LPS stimulation was tested on SMCs by time course experiments (24 h and 48 h) to identify the optimal inflammatory activity of LPS, using *IL-6* and *IL-8* expression as a readout (data not shown). The DHT concentration of 30 nM was selected as it was demonstrated to represent the physiological concentration of DHT within the human prostate (Marks *et al.* 2006) able to completely block TNFα-, LPS-, or activated CD4+ T-cell-induced inflammatory response in myofibroblast hBPH cells (Vignozzi *et al.* 2012*a*). All the experiments were performed in triplicate in at least three different cell preparations.

Immunohistochemistry cell characterization

The SMC nature of cell cultures was characterized as previously described (Comeglio *et al.* 2016). Briefly, hvSMCs and rvSMCs were seeded at the density of 1×10^4 cells on glass coverslips in GM and left untreated until complete cell adhesion. They were then immunostained using specific smooth muscle markers myosin heavy chain 11 mouse MAB (MHC11, 1:200, v/v; Abcam) and α smooth muscle actin (αSMA, 1:100 vol/vol; Sigma-Aldrich). The immunostaining for the epithelial marker cytokeratin C (pre-diluted mouse MAB; Ventana Medical System, Oro Valley, AZ, USA) was taken as negative control.

For androgen receptor (AR) detection, immunocytochemical studies were performed as previously described (Comeglio *et al.* 2016). Briefly, cells were fixed in 2% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 and blocking with 1% BSA. In rvSMCs the immunostaining was performed with anti-androgen receptor mouse MAB (1:50, v/v; Santa Cruz Biotechnology) followed by Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:200, v/v; Invitrogen Molecular Probes). In hvSMCs the anti-androgen receptor rabbit polyclonal antibody (1:50, v/v; Santa Cruz Biotechnology) was used followed by Alexa Fluor 568 goat anti-rabbit IgG (H+L) secondary antibody (1:200, v/v; Invitrogen Molecular Probes). Antibody specificity was verified by omitting the primary antibody.

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Immunofluorescence microscopy

The rvSMCs and hvSMCs were seeded at the density of 1×10^4 cells on glass coverslips in GM. After overnight serum starvation, cells were left untreated (NT) or incubated with DHT (30 nM) in the presence or absence of BICA (1 μM) for 24 h, then stimulated with LPS (100 ng/mL) for 5 h.

Immunostaining was performed as previously described (Penna *et al.* 2009) using a primary mouse MAB against NF-κB p65 (1:100, v/v; Santa Cruz Biotechnology) followed by Alexa Fluor 488 goat anti-mouse conjugated secondary antibody (1:200, v/v; Invitrogen Molecular Probes). Slides were then examined with a phase contrast microscope (Microphot-FX microscope, Nikon). Experiments were performed three times with three different cell preparations.

Analysis of cytokines and chemokines production by hvSMCs cells

The hvSMCs were seeded onto six-well plates in 1 mL of GM (1.5×10^5 cells/well) and after overnight serum starvation they were left untreated (NT) or were pre-treated for 24 h with DHT (30 nM), in the presence or absence of BICA (1 μM), and then stimulated with LPS (100 ng/mL) or IFNγ (1000 UI/mL) for further 24 h. Cell culture supernatants were collected and analyzed according to the manufacturer's instructions (Bio-Rad Laboratories).

A bead-based multiplex immunoassay (Bio-Rad Laboratories) and a Bioplex 200 system (Luminex Map Technology, Bio-Rad Laboratories) were used to simultaneously measure the concentrations of the following cytokines and chemokines in cell culture supernatants (IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IFNγ, TNFα, GCSF (granulocyte-colony-stimulating factor), GMCSF (granulocyte-macrophage colony-stimulating factor), VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), IP10 (interferon-inducible protein 10), MCP1 (monocyte chemoattractant protein-1), RANTES (regulated on activation, normal T cell expressed and secreted), MIP1A and MIP1B (macrophage inflammatory protein 1-alpha and 1-beta)). Briefly, 50 μL of supernatant were added to 50 μL of antibody-conjugated beads directed against the analytes listed above in a 96-well filter plate. After a 30-min incubation, the plate was washed and the biotinylated anti-cytokine antibody solution was added to each well. The plate was then washed and streptavidin-conjugated phycoerythrin (PE) was added to each well. After a final wash, the plate was analyzed with the Bioplex 200 system. Standard curves were derived from various concentrations of the different cytokine standards in the assay and followed the same protocol as the supernatant samples. The concentration of each cytokine (pg/mL) in each supernatant was then calculated by Bioplex software (Bio-Rad Laboratories) and normalized by the relative cell count. The assay sensitivity for all proteins tested was <1 pg/mL.

Flow cytometry

The hvSMCs were seeded onto 60 mm plates (2×10^5 cells/well) in 3 mL of GM and after serum starvation cells were pre-treated for 24 h with DHT (30 nM) and thereafter stimulated with IFNγ (1000 UI/mL) for 48 h. Untreated cells (NT) were used, as control. The hvSMCs were detached by trypsin (Sigma-Aldrich) and after centrifugation 1×10^5/mL cells for each experimental point were counted and immunolabeled with HLA-DR (PE) (1:10, v/v; BD Biosciences PharMingen) for 15 min at room temperature. Unlabelled cells were used as negative control. Flow cytometric acquisition was performed by collecting 1×10^4 events on a FACScalibur (BD Biosciences) or on Epics XL-System II flow cytometer (Beckman Coulter), and data were analyzed as previously described (Urbani *et al.* 2006).

Real-time quantitative reverse transcriptase polymerase chain reaction

Isolation of RNA from tissues was performed by solubilizing with TRIzol reagent (Life Technologies) and using chloroform thus obtaining an aqueous solution of nucleic acids.

The primary cultures smooth muscle cells were processed utilizing the a commercial RLT lysis buffer (Qiagen). Total RNA extraction for both tissues and cells was finally obtained by purification of the aqueous solutions using the RNeasy Mini Kit (Qiagen), according to the manufacturers' instructions.

cDNA synthesis was carried out using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time quantitative RT-PCR amplification and detection were carried out using SsoAdvanced Universal Probes Supermix or SsoAdvanced Universal SYBR Green Supermix, and a CFX96 Real-Time PCR Detection System (all from Bio-Rad Laboratories). PCR probes and primers were purchased from Life Technologies. Specific PCR

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primers for human and rat target genes were designed on sequences available at the National Center for Biotechnology Information GenBank (https://www.ncbi.nlm.nih.gov/genbank/) or Ensemble Genome (http://www.ensembl.org). The expression of 18S ribosomal subunit was used as the reference gene for the relative quantization of the target genes based on the comparative threshold cycle $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Results

mRNA expression of sex steroid receptors in human and rat vaginal tissues

Figure 1 shows the mRNA expression of *AR* as compared to the other sex steroid receptors in human (panel A) and rat (panel B) distal vagina tissues, by using real-time RT-PCR. In both human (panel A) and rat (panel B) vaginal tissue, *AR* mRNA shows an almost three log unit higher expression as compared to estrogen receptor β (*ERβ*, $P < 0.001$) and G-protein coupled estrogen receptor 1 (*GPER1*, $P < 0.001$), reaching an expression level that is of the same order of magnitude of the estrogen receptor α (*ERα*), although slightly lower (*ERα*, $P < 0.01$ and $P < 0.05$ vs human and rat *AR*, respectively). *AR* expression was significantly higher than that one of progesterone receptor (*PR*), but only in human vagina ($P < 0.001$, panel A). We then evaluated the expression of *AR* as compared to the other sex steroids receptors in hvSMCs and rvSMCs (Fig. 1, panels C and D, respectively). In hvSMCs, *AR* resulted the most expressed receptor among other sex steroid, receptor, including *ERα*, *ERβ*, *GPER1* and *PR* (all $P < 0.001$; Fig. 1, panel C). A similar figure was observed in rvSMCs, although, in these cells, *Ar* was less expressed than *Pr* ($P < 0.05$; Fig. 1, panel D).

Characterization of smooth muscle cells from human (hvSMCs) and rat (rvSMCs) vagina

Human vaginal smooth muscle cells (hvSMCs) showed a high positive staining for the specific smooth muscle markers myosin heavy chain 11, MHC11 (91.5 ± 6.2%; Fig. 2, panel A) and for the α smooth muscle actin, αSMA (100%; Fig. 2, panel B), while resulting negative for epithelial marker cytokeratin (Fig. 2, panel C). Rat vaginal smooth muscle cells (rvSMCs) showed a similar pattern of markers expression, with a high positive immunostaining for both smooth muscle markers (MHC11: 94.5 ± 4.4%, Fig. 2, panel D; αSMA: 90.2 ± 4.3%, Fig. 2, panel E), and negative staining for the epithelial marker cytokeratin (Fig. 2, panel F).

Figure 2 (panels G and I) shows representative images for nuclear immunostaining of AR in hvSMCs and rvSMCs, respectively, while panels H and J show the corresponding nuclear DAPI counterstaining. The quantification of cellular nuclear positivity was performed by counting positive nuclei for AR, and in both SMCs the positivity was 100%.

Toll-like receptors (TLRs) mRNA expression pattern in hvSMCs and rvSMCs

To evaluate the potential contribution of vagina SMCs to the inflammatory process, we assessed the membrane

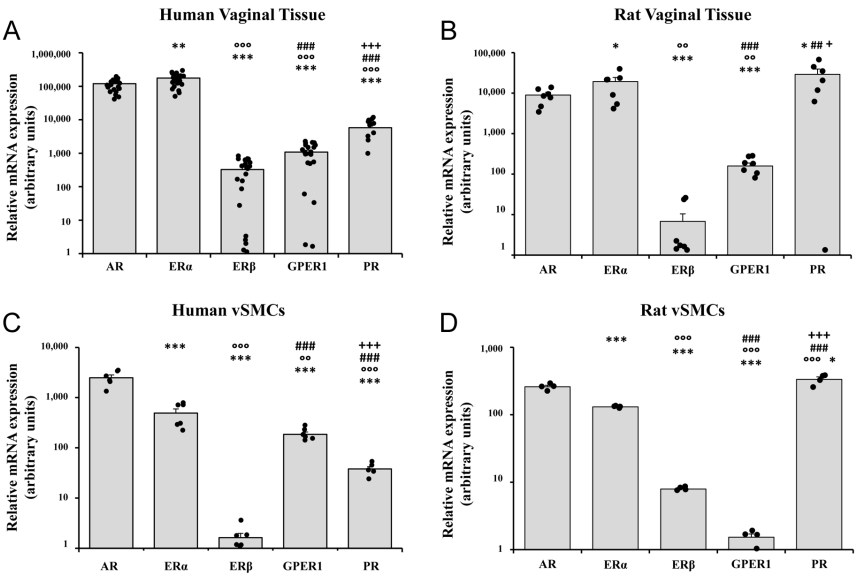

Figure 1
mRNA expression of sex steroid receptors in human and rat distal vagina tissues cells. Panels A and B show the mRNA expression, reported as scatter plot and bar graph, of sex steroid receptors *AR*, *ERα*, *ERβ*, *GPER1* and *PR* in human and rat distal vagina tissues, the former derived from laparoscopic biopsies, respectively. Panels C and D show the mRNA expression of sex steroid receptors *Ar*, *Erα*, *Erβ*, *Gper1* and *Pr* in human distal vagina and in rat distal vagina SMCs, respectively. Data were calculated according to the $2^{-\Delta\Delta Ct}$ comparative method, using ribosomal subunit 18S as the reference gene for normalization (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs AR; °°$P < 0.01$, °°°$P < 0.001$ vs ERα; ##$P < 0.01$, ###$P < 0.001$ vs ERβ; $^{+}P < 0.05$, $^{+++}P < 0.001$ vs GPER1).

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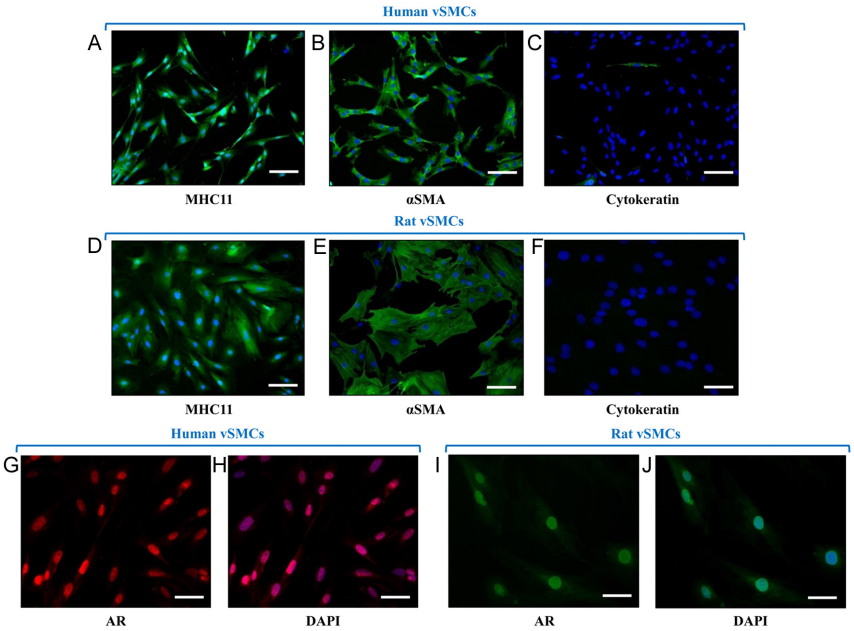


Figure 2
Characterization of SMCs isolated from human and rat vagina tissues. Panels A, B and C show the results of hvSMCs immunostaining for specific smooth muscle markers MHC11 and αSMA, and for epithelial marker cytokeratin (taken as negative control), respectively. Panels D–F show the results of rvSMCs immunostaining for specific smooth muscle markers MHC11 and αSMA, and for epithelial marker cytokeratin (taken as negative control), respectively. The quantitative analysis was performed by counting positive cells in at least 10 fields per slide of three different cell preparations (scale bar = 100 µm). Panels G and H show two representative images of hvSMCs nuclear immunostaining for AR receptor and the corresponding nuclear DAPI double labeling, respectively (scale bar = 50 µm). Similarly, panels I and J show two representative images of rvSMCs nuclear immunostaining for AR receptor and the corresponding nuclear DAPI double labeling, respectively (scale bar = 50 µm). A full color version of this figure is available at https://doi.org/10.1530/JME-20-0147.

profile of toll-like receptors (TLRs), by using real-time RT-PCR for hvSMCs and rvSMCs (Fig. 3, panels A and B, respectively). In hvSMCs, we also compared the pattern of *TLR*s mRNA expression to that of primary cell lines isolated from benign prostatic hyperplasia (hBPH) samples, a well-validated model of non-professional APCs that constitutively expressed all *TLR*s (Penna *et al*. 2009) (Fig. 3, panel A). hvSMCs expressed all *TLR*s, with *TLR1, TLR2, TLR3*, and *TLR4* being the most abundant. The mRNA expression profile presented by hvSMCs was rather similar to that of hBPH cells. In particular, hvSMCs showed a significantly higher expression of *TLR2* ($P < 0.001$), *TLR5* ($P < 0.001$) and *TLR9* ($P < 0.01$), and a significantly lower expression of *TLR1* ($P < 0.001$), *TLR3* ($P < 0.001$) and *TLR6* ($P < 0.01$), when compared to hBPH cells (Fig. 3, panel A). Similarly, rvSMCs expressed all TLRs, with a relatively higher level of transcripts encoding *Tlr1, Tlr2, Tlr3, Tlr4* and *Tlr10* (Fig. 3, panel B).

Treatment with the selective AR agonist, dihydrotestosterone (DHT) inhibits basal and LPS-induced mRNA expression and secretion of pro-inflammatory mediators in hvSMCs

Under basal (untreated, NT) conditions, hvSMCs produced several cytokines, chemokines, and growth factors in the culture medium (Fig. 4). Among these factors, MCP1 was the most abundantly secreted, followed from, by at

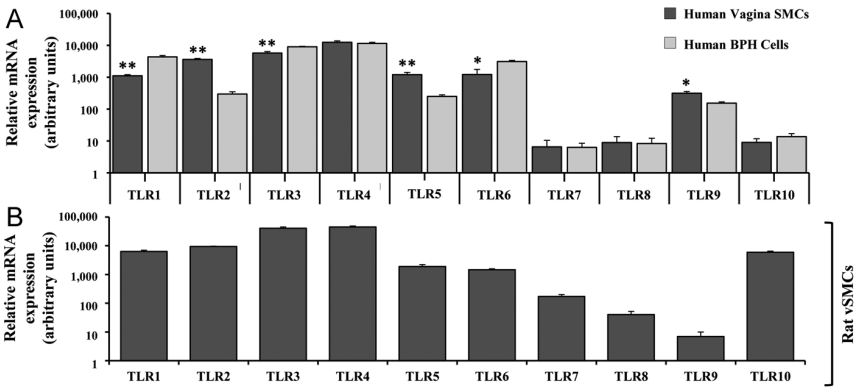
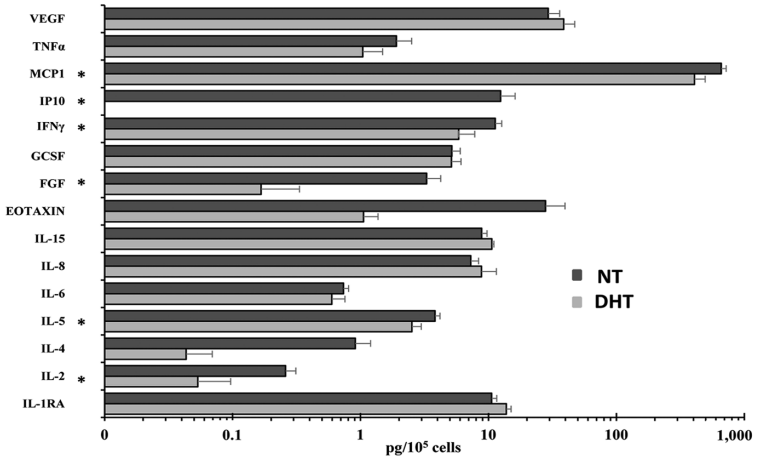


Figure 3
Toll-like receptors (*TLR*s) mRNA expression pattern and anti-inflammatory effect of DHT in vSMCs. Panel A shows the complete panel of the *TLR*s mRNA expression in hvSMCs, with the relative *TLR*s gene expression in human BPH cells taken as positive control. The results were calculated according to the $2^{-\Delta\Delta Ct}$ comparative method, using the ribosomal subunit 18S as the reference gene for normalization, and data are represented as the mean ± S.E.M. of three independent experiments (*$P < 0.01$, **$P < 0.001$ vs hBPHs). Panel B shows the complete panel of *Tlr*s mRNA expression in rvSMCs. Data were calculated according to the $2^{-\Delta\Delta Ct}$ comparative method, using the ribosomal subunit 18S as the reference gene for normalization and are represented as the mean ± S.E.M. of three independent experiments.



	NT (pg/10⁵ cells)	DHT (pg/10⁵ cells)
VEGF	29.32 ± 6.75	38.66 ± 8.64
TNFα	1.91± 0.60	1.04 ± 0.45
MCP1	659.85 ± 62.56	407.85 ± 86.15*
IP10	12.44 ± 3.74	0.00*
IFNγ	11.28 ± 1.42	5.86 ± 1.95*
GCSF	5.18 ± 0.85	5.14 ± 0.96
FGF	3.28 ± 0.96	0.17 ± 0.17*
EOTAXIN	27.97 ± 11.65	1.06 ± 0.31
IL-15	8.85 ± 0.90	10.62 ± 0.39
IL-8	7.28 ± 1.09	8.81 ± 2.73
IL-6	0.74 ± 0.07	0.60 ± 0.16
IL-5	3.82 ± 0.36	2.52 ± 0.46*
IL-4	0.91 ± 0.29	0.04 ± 0.03
IL-2	0.26 ± 0.05	0.05 ± 0.04*
IL-1RA	10.58 ± 1.04	13.78 ± 1.25

Figure 4
The effect of DHT pre-treatment on basal production of pro-inflammatory cytokines, chemokines, and growth factors in hvSMCs culture supernatant. The data represent the mean ± S.E.M. of three independent experimental points performed in triplicate (*P < 0.05 vs NT). The numerical values expressed as pg/10^5 cells for each cytokine/chemokine are reported in the Table.

least one log unit, VEGF, eotaxin, IP10 and IFNγ (Fig. 4). Interestingly, pre-treatment with the selective AR ligand dihydrotestosterone (DHT) significantly blunted basal secretion of MCP1, also significantly inhibiting IP10, IFNγ, FGF, IL-5 and IL-2 basal secretion (Fig. 4).

We next evaluated the effect of DHT (30 nM for 24 h), with or without the non-steroidal AR antagonist bicalutamide (1 μM for 24 h), on LPS-stimulated cytokine production in hvSMCs (Fig. 5, panels A–K). LPS (100 ng/mL for 24 h) significantly increased the secretion

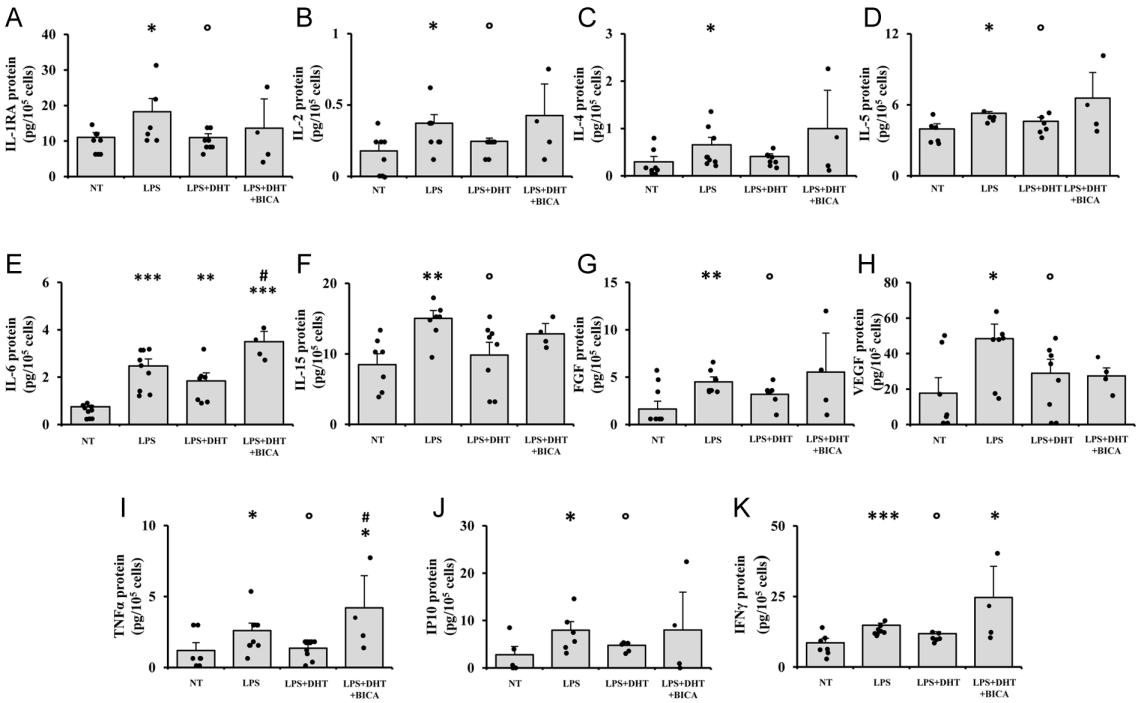


Figure 5
DHT effects on secretion of pro-inflammatory mediators in LPS-stimulated vaginal hSMCs. The hvSMC supernatants from each experimental point were analyzed for the production of the indicated proinflammatory cytokines, chemokines, and growth factors (panels A–K). The data represent the mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (*P < 0.05, **P < 0.01, ***P < 0.001 vs NT; °P < 0.05 vs LPS; #P < 0.05 vs LPS + DHT).

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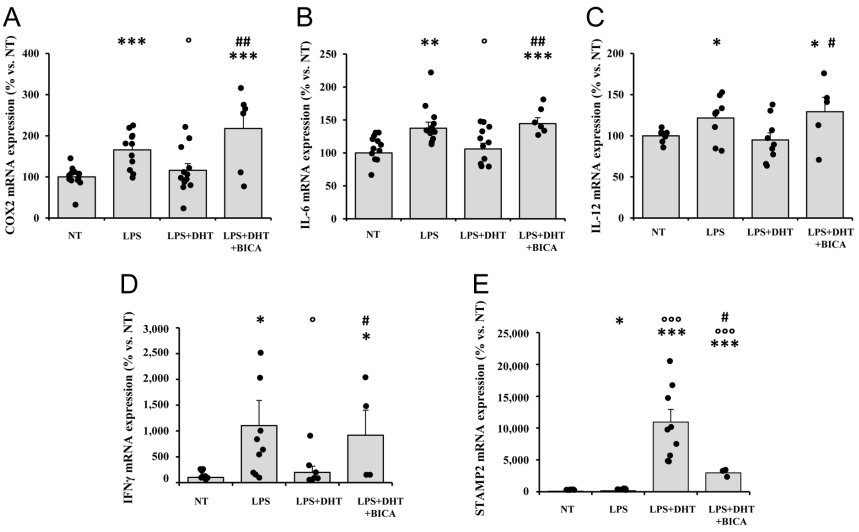

Figure 6
DHT effects on mRNA expression of pro-inflammatory mediators in LPS-stimulated hvSMCs. Human vaginal SMCs were left untreated (NT) or stimulated with LPS, in the presence or absence of DHT pre-incubation (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The mRNA expression of pro-inflammatory markers *COX2, IL-6, IL-12* and *IFNγ* are shown in panels A–D, respectively, whereas anti-inflammatory and androgen-sensitive *STAMP2* mRNA expression is shown in panel E. Data were calculated according to $2^{-\Delta\Delta Ct}$ comparative method, using 18S ribosomal subunit as reference gene for normalization. Results are expressed as percentage of NT and are reported as mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (**P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs NT; °*P* < 0.05, °°°*P* < 0.001 vs LPS; #*P* < 0.05, ##*P* < 0.01 vs LPS + DHT).

of several pro-inflammatory proteins, including IL-2, IL-4, IL-5, VEGF, TNFα, IP10 (all $P < 0.05$ vs NT), IL-15 and FGF (both $P < 0.01$ vs NT), IL-6 ($P < 0.001$ vs NT) and, most importantly, IFNγ ($P < 0.001$ vs NT), while inducing a slight increase of the anti-inflammatory protein IL-1RA ($P < 0.05$ vs NT). Pre-treatment with DHT significantly counteracted the secretion of IFNγ ($P < 0.05$ vs LPS; Fig. 5, panel K), along with IL-2, IL-5, IL-15, FGF, VEGF, TNFα, and IP10 (all $P < 0.05$ vs LPS; Fig. 5, panels B, D, F, G, H, I, J, respectively), but not IL-6, for which DHT induces a decrease in secretion to the limit of significance ($P = 0.09$ vs LPS; Fig 5, panel E). A significant reduction of the anti-inflammatory cytokine IL-1RA was also observed (Fig. 5, panel A). Co-treatment of DHT with bicalutamide tended to counteract most of the effects of DHT on LPS-stimulated secretions (Fig. 5, panels A–K). In particular, TNFα and IFNγ were significantly increased by bicalutamide co-treatment, reaching a level even significantly higher than NT (Fig. 5, panels I and K, respectively), whereas VEGF secretion was unchanged by bicalutamide co-treatment (Fig. 5, panel H). Secretion of IL-8, GCSF, GMCSF, MCP1 and MIP1B was also significantly increased by LPS-treatment (all $P < 0.05$ vs NT), but neither DHT nor bicalutamide were able to affect it (data not shown).

As shown in Fig. 6 (panels A–D), LPS also significantly upregulated the mRNA expression of the inducible form of cyclooxygenase-2 (*COX2*; $P < 0.001$ vs NT), along with other inflammatory factors, namely *IL-6* ($P < 0.01$ vs NT), *IL-12* ($P < 0.05$ vs NT), and *IFNγ* ($P < 0.05$ vs NT). The expression of all these genes was significantly reduced by pre-treatment with DHT (all $P < 0.05$ vs LPS; Fig. 6, panels A–D), and co-treatment with bicalutamide completely blunted the DHT-induced effects ($P < 0.01$ vs LPS+DHT for *COX2* and *IL-6*, $P < 0.05$ vs LPS+DHT for *IL-12* and *IFNγ*; Fig. 6, panels A–D). DHT treatment also significantly upregulated the mRNA expression of the anti-inflammatory, and androgen-sensitive, gene *STAMP2* ($P < 0.001$ vs both NT and LPS), which was also stimulated by LPS, but to a much lower extent ($P < 0.05$ vs NT) (Fig. 6, panel E). Co-treatment with bicalutamide robustly counteracted DHT-induced *STAMP2* upregulation ($P < 0.05$ vs LPS+DHT; Fig. 6, panel E).

DHT inhibits NF-κB p65 nuclear translocation in LPS-stimulated hvSMCs

To better characterize the effect of DHT on the inflammatory response in LPS-stimulated hvSMCs, we evaluated nuclear translocation of NF-κB p65 by immunofluorescence (Fig. 7, panels A–E). In untreated (NT) hvSMCs, NF-κB p65 was only detected within the cytoplasm (Fig. 7, panels A and E), while LPS treatment induced a significant increase of its translocation to nuclei (21.6 ± 1.8%; $P < 0.001$ vs NT; Fig. 7, panels B and E). Co-treatment with DHT significantly blunted LPS-induced nuclear translocation of NF-κB p65, without normalizing it (11.8 ± 1.3%; $P < 0.001$ vs LPS; $P < 0.001$ vs NT; Fig. 7, panels C and E). Co-treatment with bicalutamide (1 μM for 24 h) significantly counteracted DHT effect (25.5 ± 2.1%; $P < 0.01$ vs LPS+DHT; Fig. 7, panels D and E).

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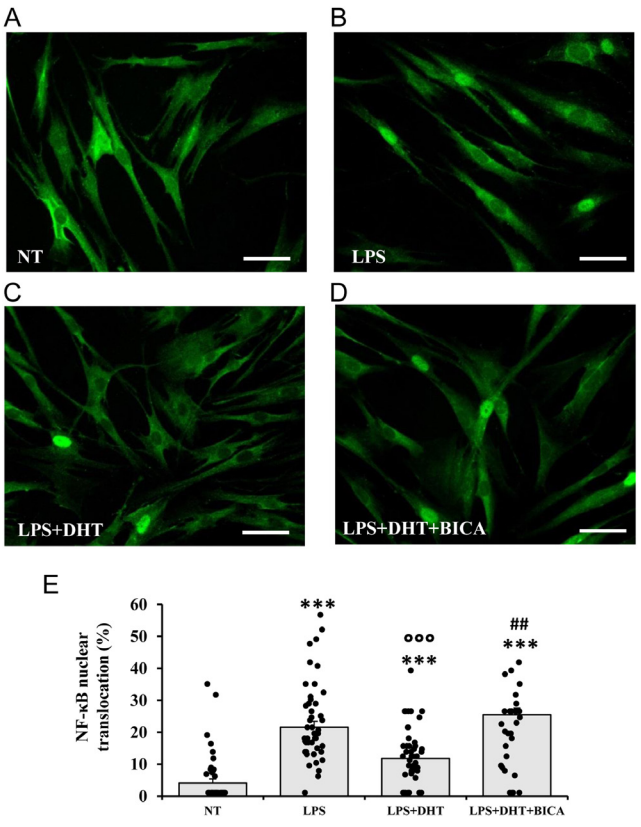

Figure 7
DHT effects on NF-κB p65 nuclear translocation in LPS-stimulated hvSMCs. Vaginal hSMCs were left untreated (NT) or stimulated with LPS (100 ng/mL) for 5 h, with or without pre-treatment with DHT (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The NF-κB p65 nuclear translocation was analyzed by immunofluorescence and representative images of NF-κB immunostaining of three experiments are shown in panels A–D for NT, LPS, LPS + DHT and LPS + DHT + BICA experimental groups, respectively (scale bar = 100 μm). Panel E bar graph shows the quantitative analysis of NF-κB nuclear translocation (evaluated in at least in 10 fields per slide), expressed as the number of positive cells as percentage of total cells. The data represent the mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (***$P < 0.001$ vs NT; °°°$P < 0.001$ vs LPS; ##$P < 0.01$ vs LPS + DHT). A full color version of this figure is available at https://doi.org/10.1530/JME-20-0147.

Treatment with DHT inhibits IFNγ-induced mRNA expression and secretion of pro-inflammatory mediators in hvSMCs

We tested the effect of the Th1 driven inflammatory cytokine, IFNγ, on hvSMCs. Stimulation with IFNγ (1000 IU/mL for 24 h) significantly increased the mRNA expression of several inflammatory factors such as *iNOS*, *COX2*, *MCP1* and *IL-12* (all $P < 0.001$ vs NT; Fig. 8, panels A-D). In particular, IFNγ induced a ~3-log unit increase of *IP10* mRNA expression ($P < 0.001$ vs NT; Fig. 8, panel E). All these effects were significantly blunted by DHT pre-treatment (all $P < 0.05$ vs IFNγ; Fig. 8, panels A-E).

Figure 9 (panels A–F) shows that IFNγ stimulation (1000 IU/mL for 24 h) increased the secretion of several pro-inflammatory chemokines and cytokines (IL-1β, IL-2, IL-5, IL-6, MCP1 and GCSF, all $P < 0.001$ vs NT), and DHT pre-treatment (30 nM for 24 h) partially blunted it (all $P < 0.05$ vs IFNγ). Interestingly, DHT pre-treatment also reduced, even though without reaching statistical significance, IFNγ-stimulated secretion of TNFα, VEGF, FGF and IL-17 (data not shown). Finally, IFNγ induced a significant increase in the secretion of IL-1RA, IL-4, IL-7, IL-9, IL-12, IL-13, IL-15, IP10, MIP1A, MIP1B and RANTES; however, no significant effects were observed in their levels after DHT pre-treatment (data not shown).

Evaluation of the effects of IFNγ and DHT on the HLA-DR expression in hvSMCs

We next evaluated the effect of IFNγ (1000 UI/mL for 48 h) with or without DHT pre-treatment on the expression of class II histocompatibility (HLA-DR) antigens on the cell surface of hvSMCs, assessed by using flow cytometry (Fig. 9, panel G). As expected, HLA-DR was not expressed in NT and DHT only-treated cells, whereas its expression was significantly increased (47.5 ± 5.5%; $P < 0.001$ vs NT) after IFNγ stimulation (Fig. 9, panels G and H). Pre-treatment with DHT significantly decreased IFNγ-induced HLA-DR expression (37.6 ± 1.3%; $P < 0.01$ vs IFNγ), albeit without normalizing it ($P < 0.001$ vs NT) (Fig. 9, panels G and H).

Anti-inflammatory effect of the selective AR agonist, dihydrotestosterone (DHT) in rvSMCs

Some of the experiments performed in hvSMCs were repeated in rvSMCs. The effect of DHT (30 nM for 24 h), in presence or absence of bicalutamide (1 μM for 24 h), was assessed in rvSMCs, under basal (Fig. 10, panels A–F) and after LPS-stimulated condition (Fig. 10, panels G-J). DHT significantly inhibited the basal mRNA expression of the proinflammatory markers *Il-6* ($P < 0.01$ vs NT), *Cxcl1* ($P < 0.001$ vs NT), *Mcp1* ($P < 0.05$ vs NT) and *Cox2* ($P < 0.001$ vs NT) (Fig. 10, panels A, B, D and E, respectively), while significantly increasing the mRNA expression of the anti-inflammatory and androgen-dependent factor *Stamp2* ($P < 0.01$ vs NT; Fig. 10, panel F). In most but not all cases, DHT effect was blunted by co-treatment with bicalutamide ($P < 0.05$ vs DHT for *Il-6* and *Mcp1*; $P < 0.001$ vs DHT for *Cox2*; Fig. 10, panels A, D and E, respectively).

We also evaluated the effect of increasing concentrations of DHT on rvSMCs stimulated by

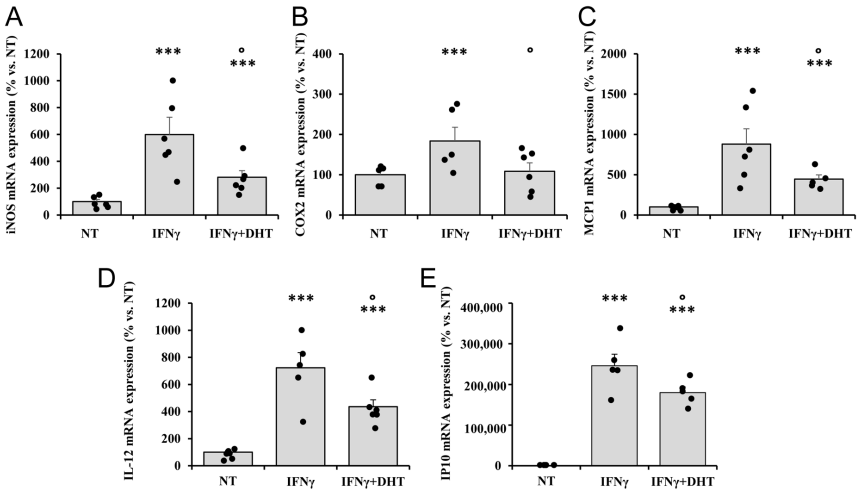

Figure 8
DHT effects on mRNA expression of pro-inflammatory mediators in INFγ-stimulated hvSMCs. Panels A–E report the mRNA expression analysis of pro-inflammatory cytokines and chemokines after hvSMC cells were stimulated with IFNγ (1000 IU/mL) for 24 h, with or without DHT incubation (30 nM, 24 h). Data were calculated according to $2^{-\Delta\Delta Ct}$ comparative method, using 18S ribosomal subunit as reference gene for normalization. Results are expressed as percentage of untreated cells (NT) and are reported as mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (***$P < 0.001$ vs NT; °$P < 0.05$ vs IFNγ).

LPS (Fig. 10, panel G). Increasing concentrations of DHT (ranging from 10 pM to 1 μM) significantly blunted LPS-induced *Il-6* and *Cxcl1* mRNA expression in a dose-dependent manner with a similar IC_{50} ($IC_{50} = 5.1 \times 10^{-10}$ M for *Il-6*, and $IC_{50} = 5.6 \times 10^{-10}$ M for *Cxcl1*) and maximum inhibitory effect ($I_{max} = 82.1 \pm 9\%$ for *Il-6*, and $I_{max} = 78.2 \pm 7.2\%$ for *Cxcl1*; Fig. 10, panel G). In addition, LPS induced the mRNA expression of other pro-inflammatory mediators, namely *Il-1β*, *Mcp1* (both $P < 0.001$ vs NT; Fig. 10, panels H and I, respectively) and *Cox2* ($P < 0.01$ vs NT; Fig. 10, panel J). LPS effects were significantly blunted by DHT (all $P < 0.05$ vs LPS;

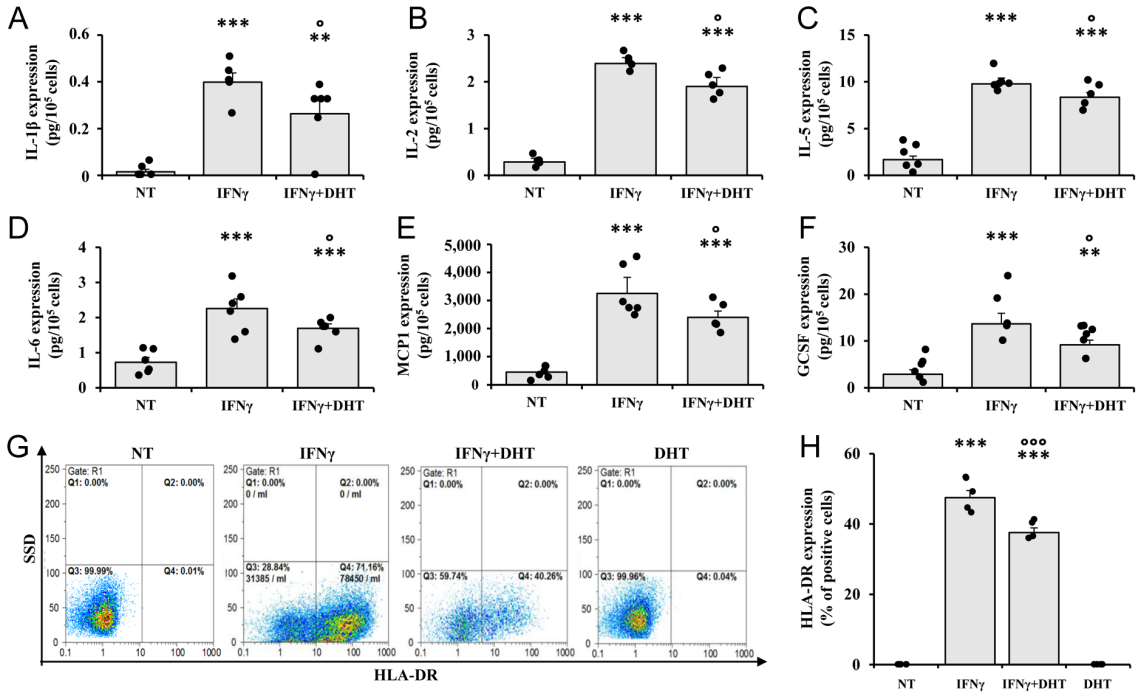

Figure 9
DHT effects on the secretion of pro-inflammatory mediators and on the cell surface HLA-DR expression in IFNγ-stimulated hvSMCs. Vaginal hSMCs supernatants from each experimental point were analyzed for the secretion of pro-inflammatory cytokines, chemokines and growth factors (panels A–F). Data are represented as the mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (**$P < 0.01$, ***$P < 0.001$ vs NT; °$P < 0.05$ vs IFNγ). Panel G reports the results of a representative experiment of flow cytometry showing the expression of HLA-DR in hvSMCs stimulated with IFNγ (1000 IU/mL) for 48 h and/or of DHT treatment (30 nM, for 24 h). Panel H shows the quantification of cell positivity for HLA-DR, expressed as percentage of positive cells compared to the total number of cells. Data are reported as mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (***$P < 0.001$ vs NT; °°°$P < 0.001$ vs IFNγ). A full color version of this figure is available at https://doi.org/10.1530/JME-20-0147.

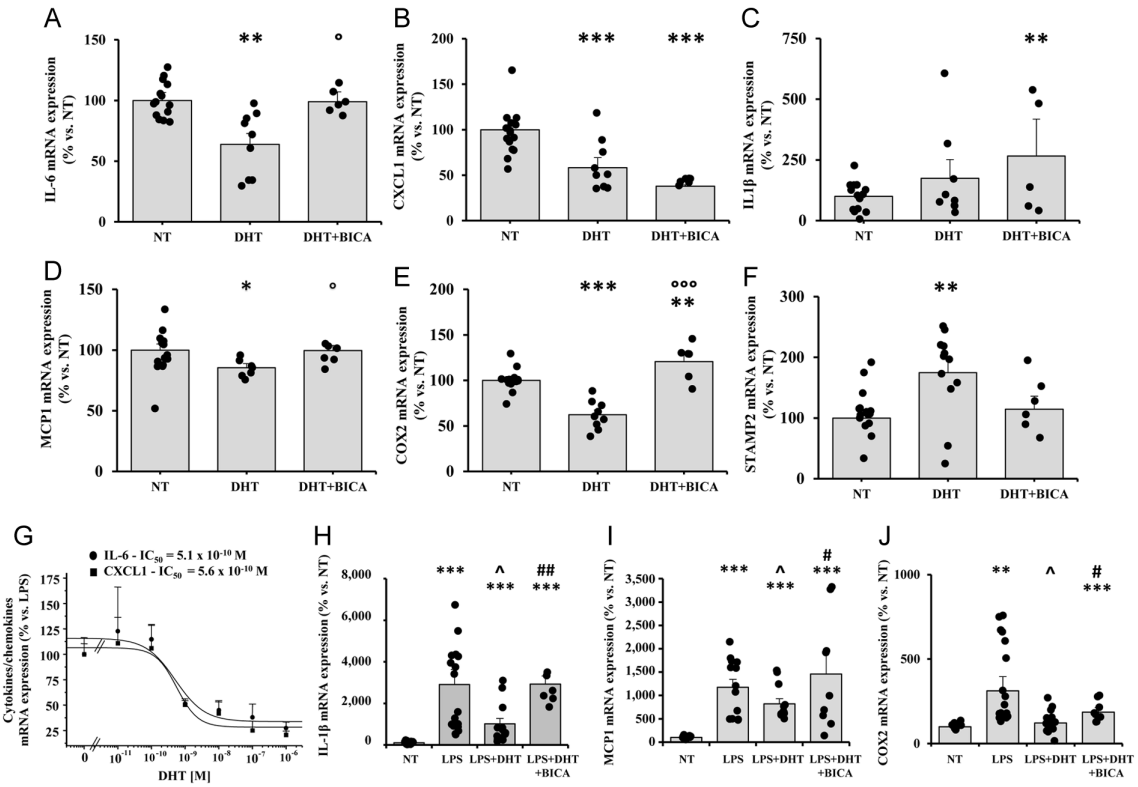

Figure 10

DHT effects on basal and LPS-induced mRNA expression of proinflammatory and androgen-dependent markers in rvSMCs. Rat vaginal SMCs were cultured for 24 h in serum-free medium alone (untreated cells, NT) or DHT (30 nM), with or without anti-androgen bicalutamide (BICA, 1 μM) or were stimulated with LPS (100 ng/mL for 24 h), with or without pre-incubation with DHT and in the presence or absence of BICA The mRNA expression of pro-inflammatory (*Il-6*, *Cxcl1*, *Il-1β*, *Mcp1*, *Cox2*) and androgen-dependent (*Stamp2*) markers after pre-incubation with DHT in combination or not with bicalutamide is showed in panels A–F. Panel G shows the inhibitory effect of increasing concentrations of DHT on LPS-induced mRNA expression of *Il-6* and *Cxcl1* in rvSMCs. Ordinate: mRNA expression of the indicated pro-inflammatory factors (black square: *Cxcl1*; black circle: *Il-6*) induced by LPS after DHT pre-incubation. Data are expressed as percentage of the effect of LPS alone. Abscissa: molar concentrations of DHT (10 pM–1 μM). Data are represented as the mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph. The relative half-maximal response (IC_{50}) value is reported in the graph. Panels H–J show the mRNA expression of pro-inflammatory markers *Il-1β*, *Mcp1*, *Cox2*, induced by LPS stimulation alone and after pre-incubation with DHT, in combination or not with bicalutamide. Data were calculated according to the $2^{-\Delta\Delta Ct}$ comparative method, using the 18S ribosomal subunit as the reference gene for normalization. The results represent the mean ± S.E.M. of three independent experiments, performed in triplicate and reported as percentage vs untreated cells (NT) (**P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs NT; °*P* < 0.05, °°°*P* < 0.001 vs DHT, ^*P* < 0.05 vs LPS; #*P* < 0.05, ##*P* < 0.01 vs LPS + DHT).

Fig. 10, panels H-J), while co-treatment with bicalutamide completely abolished the inhibitory effects of DHT (*P* < 0.01 vs LPS+DHT for *Il-1β*, *P* < 0.05 vs LPS+DHT for *Mcp1* and *Cox2*; Fig. 10, panels H, I and J, respectively).

DHT inhibits NF-κB p65 nuclear translocation in LPS-stimulated rvSMCs

Immunofluorescence analyses for NF-κB p65 nuclear translocation in rvSMCs are shown in Fig. 11 (panels A–E). Similarly to hvSMCs, in untreated (NT) rvSMCs, NF-κB p65 was detected only within the cytoplasm (Fig. 11, panels A and E). LPS stimulation (100 ng/mL for 5 h) induced a partial but significant translocation of NF-κB p65 to the nucleus (40.2 ± 2.2%; *P* < 0.001 vs NT; Fig. 11, panels B and E), which was significantly inhibited (20.8 ± 1.9%; *P* < 0.001 vs LPS), albeit not normalized (*P* < 0.001 vs NT), by DHT treatment (Fig. 11, panels C and E). Co-treatment with bicalutamide (1 μM for 24 h) partially contrasted DHT-induced effect (31.3 ± 2.3%; *P* < 0.05 vs LPS; *P* < 0.01 vs LPS+DHT; Fig. 11, panels D and E).

Discussion

The present study demonstrates, for the first time, that human vagina smooth muscle cells (hvSMCs) might be actively involved in the inflammatory response, since they express all TLRs and secrete several pro-inflammatory factors upon stimulation with LPS – used to mimic an infectious-driven inflammation – and IFNγ – used to

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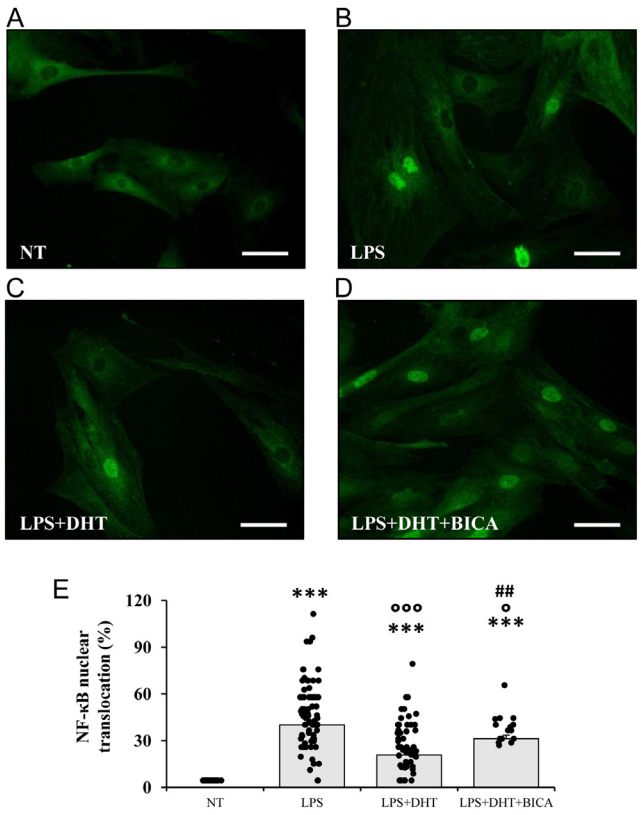

Figure 11
DHT effects on LPS-induced NF-κB p65 nuclear translocation in rvSMCs. Rat vaginal SMC cells were stimulated with LPS (100 ng/mL for 5 h) with or without pre-incubation with DHT (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The NF-κB p65 activation in rvSMCs was quantified by immunofluorescence analysis. The representative images in panels A–D show the results of NF-κB immunostaining in each experimental group (scale bar = 50 μm). In panel E, the bar graph reports the number of cells with NF-κB nuclear positivity (evaluated in at least in 10 fields per slide), expressed as percentage of total cells. The data represent the mean ± S.E.M. of three independent experiments performed in triplicate and are reported as scatter plot and bar graph (***$P < 0.001$ vs NT; °$P < 0.05$, °°°$P < 0.001$ vs LPS; ##$P < 0.01$ vs LPS + DHT). A full color version of this figure is available at https://doi.org/10.1530/JME-20-0147.

mimic a Th1-driven immune response. The hvSMCs were indeed able to respond to LPS by secreting several cytokines and chemokines, including those related to the Th1-chronic immune response such as IFNγ, IP10, IL-2, IL-12, and IL-15 (Lees 2015). Stimulation of hvSMCs with IFNγ, in turn, substantially increased the production of several inflammatory agents and induced the expression of HLA-DR on hvSMCs surface. *De novo* expression of HLA-DR, a particular human leukocyte antigens (HLA) molecule that is unknown in conventional SMCs (Erokhina *et al.* 2018), indicated that, under inflammatory conditions, hvSMCs acquire the ability to take part in antigen presentation and in persistent chronic inflammation. More importantly, we also found that hvSMCs expressed high levels of AR, through which DHT counteracts either LPS- or IFNγ-induced inflammation by blunting the expression and secretion of inflammatory factors, including IFNγ itself and IP10, the two most important Th1-cytokines (Dufour *et al.* 2002). DHT also significantly counteracted IFNγ-induced expression of HLA-DR on the cell surface. These findings, derived from a large set of in vitro experiments, consistently indicated that hvSMCs might behave as resident non-professional APCs that, after an acute bacterial infection (experimentally mimicked by LPS stimulation), promote a Th1-polarized immune response and chronicity (Rose 2001). Noteworthy, the activation of AR by DHT exerts a relevant anti-inflammatory effect by blunting the ability of hvSMCs to respond to inflammatory stimuli and to perpetuate inflammation. Accordingly, similar anti-inflammatory effects had been previously demonstrated in male prostatic tissue (Vignozzi *et al.* 2012*a*, 2013*a*). Even though some subtle differences were observed, the similarities of results obtained in rat and human vagina smooth muscle suggest that both the ability of vSMCs to take part to the inflammatory response and the anti-inflammatory role of DHT are, most likely, not species-specific.

Inflammation usually represents the main immune system's defense to harmful stimuli, aimed at removing pathogenic *noxae* and at restoring tissue homeostasis (Chen *et al.* 2017*b*). Under most conditions, APCs play a relevant role in the local immune surveillance by allowing the host to distinguish between self- vs non-self, as well as pathogenic vs commensal bacteria (Medzhitov 2001). However, in some conditions, an exacerbated activation of APCs, which continues even after the removal of pathogenic microorganisms, polarizes lymphocytes toward a Th1 phenotype, with the consequent production of the Th1 cell-specific cytokines (i.e. IFNγ) (Chen *et al.* 2017*b*). This process leads to the break in the tolerance to self-antigens and to a progression from an acute toward a chronic immune-mediated local disease (Chen *et al.* 2017*b*). Noteworthy, these events have been also crucially involved in the development of many diseases of female genitourinary tract, including dyspareunia (Farage *et al.* 2008), vulvodynia (Zanotta *et al.* 2018), vestibulodynia (Tommola *et al.* 2016), endometriosis (Symons *et al.* 2018), as well as cystitis (Singh *et al.* 2013).

On the other hand, both animal and human studies have been extensively documenting that androgens act as important anti-inflammatory factors (Gilliver 2010). With regard to genitourinary tissue in males, we previously demonstrated the ability of human prostatic stromal

cells (hBPH cells) to act as non-professional APCs, and to actively contribute to intraprostatic chronic inflammatory diseases (Penna *et al.* 2009, Vignozzi *et al.* 2012*a*, 2013*a*). In this model, we showed a prominent anti-inflammatory effect of the AR agonist DHT in inhibiting the potential of hBPHs to induce and sustain inflammatory responses (Vignozzi *et al.* 2012*a*). Although emerging literature suggested that androgen-dependent pathway(s) might be involved in the resolution of genitourinary conditions in women, such as the GSM (Simon *et al.* 2018), immunomodulatory effects of androgen signaling in the vagina was not investigated so far.

Therefore, in the first set of experiments, we isolated and characterized SMCs derived from human and rat vagina, to ascertain potential species-specific biological effects. Both hvSMCs and rvSMCs lines express all sex steroids receptors, with an overall abundance of AR. In particular, in isolated hvSMCs, *AR* resulted the most expressed receptor, with mRNA expression levels significantly higher as compared to *ERα, ERβ, GPER1* and *PR*. In contrast, when the human vaginal tissue homogenates was analyzed, *AR* was abundantly expressed, but reaching a level significantly lower than *ERα*. A similar pattern of expression was also observed in rvSMCs and rat vaginal tissue. Accordingly, previous immunohistochemistry analyses in the monkey and rat vagina demonstrated that the highest proportion of AR-immunoreactive cells occurred in the *muscularis* and lamina propria (Pessina *et al.* 2006, Bertin *et al.* 2014), whereas ERα and PR seem to be mostly expressed in the epithelium (Pessina *et al.* 2006). This is consistent with the fact that, in our study, AR resulted less expressed than ERα and PR when analyzing the whole tissue, which obviously includes all the other cellular components such as the epithelial one.

Noteworthy, either hvSMCs or rvSMCs also expressed all identified *TLR*s, with hvSMCs showing a similar expression pattern to that found in hBPHs, a well-validated non-professional APCs (Penna *et al.* 2009), thus indicating that also hvSMCs might have the ability to initiate and coordinate an acute reaction to pathogens. *TLR1–4* emerged as the most expressed, with *TLR2* being found at even higher level than in hBPHs. TLR4 is considered a crucial defense against not only viruses and protozoa, but also bacteria, thanks to its binding to LPS of Gram-negative species (Nasu & Narahara 2010). TLR2 also recognizes several key molecules on the surface of pathogens, such as LPS, or lipoproteins and peptidoglycans that are commonly released during genital tract infections (Shey *et al.* 2016).

We also demonstrated that, in basal conditions, hvSMCs secrete several proinflammatory and growth factors, including MCP1, VEGF, eotaxin, IL-8, IL-15, IP10 and IFNγ. Pre-treatment with the potent and selective AR agonist DHT markedly reduced this basal secretion. In addition, we tested the effect of DHT in hvSMCs triggered by two canonical inflammatory stimuli: LPS, used to mimic an infection (Rusek *et al.* 2018), and IFNγ, used to simulate a chronic and self-perpetuating inflammation (Hamidzadeh *et al.* 2017). After LPS stimuli, hvSMCs increased the secretion of acute (i.e. IL-2, IL-4, IL-5, IL-15, TNFα and IL-1RA) and Th1-related cytokines (i.e. IFNγ and IP10), along with growth factors (FGF and VEGF). DHT significantly counteracted the effects of LPS, restoring cytokines secretion down to basal levels. Pre-treatment with DHT significantly decreased also LPS-induced mRNA expression of inflammatory genes such as *COX2, IL-6, IL-12A* and *IFNγ*, while upregulating the anti-inflammatory and androgen-sensitive gene *STAMP2*. Co-treatment with the AR antagonist bicalutamide was able to dampen almost all these effects, thus indicating that the observed anti-inflammatory activity of DHT was mainly mediated by AR. Interestingly, bicalutamide did not significantly antagonize the effect of DHT on VEGF secretion. This could be explained with the fact that bicalutamide has been reported to be ineffective in blocking non-genomic activation of the AR, and that non-genomic signaling of the AR is involved in a positive modulation of proliferation (Leung & Sadar 2017). The anti-inflammatory effect of DHT on IFNγ secretion is of particular relevance, since IFNγ is the most potent Th1-cytokine (Liblau *et al.* 1995), and its signaling plays a key role in development of autoimmunity and chronic, self-perpetuating inflammatory diseases (Chen *et al.* 2017*a*). In rvSMCs, we found similar anti-inflammatory effects of DHT on LPS-induced cytokines and chemokines mRNA expression and secretion into the media. Noteworthy, in this cell culture, increasing concentrations of DHT exerted a dose-dependent reduction of LPS-induced cytokines and chemokines' expression with an IC_{50} ~ 0.5 nM, a value close to the physiological concentrations of DHT in women (Rothman *et al.* 2011).

The mechanisms by which androgen signaling exerts its anti-inflammatory effects are not completely understood. In previous studies in human BPH cells, we demonstrated that DHT inhibits nuclear translocation and activation of NF-κB, a master transcription factor in inflammation (Tak & Firestein 2001, Vignozzi *et al.* 2012*a*). The same effect was also described in a modified adipocyte chronic inflammation model

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(Morooka *et al.* 2016). Similarly, in the present study, inhibition of LPS-induced nuclear translocation of NF-κB by DHT was detected in both rvSMCs and hvSMCs.

The effect of DHT was also tested after treatment of vSMCs with IFNγ. Pre-treatment with DHT counteracted IFNγ-induced mRNA expression of *iNOS, COX2, MCP1, IL-12* and *IP10*, which are all involved in amplifying and sustaining inflammatory states (Barrios-Rodiles *et al.* 1999, Proost *et al.* 2003, Mitchell *et al.* 2012, Steinman 2013, Lees 2015). In particular, IP10 is a main driver toward chronic inflammatory diseases and tissue remodeling (Neville *et al.* 1997). DHT also significantly counteracted IFNγ-induced secretion of cytokines, chemokines and growth factors (IL-1β, IL-2, IL-5, IL-6, MCP1 and GCSF) in hvSMCs, as well as blunted IFNγ-induced *de novo* expression of HLA-DR on hvSMCs' surface. Overexpression of HLA-DR that occurs on non-inflammatory cells is one of the main pathogenic mechanisms observed in autoimmune diseases (Bottazzo *et al.* 1983, Jacobson *et al.* 2008). Our observations that HLA-DR may be experimentally induced on hvSMCs indicate their capacity to present autoantigens to T lymphocytes. It is conceivable that, after the first phases of the host reaction (mimicked by LPS stimulation), the consequent production of Th1 cytokines, including IFNγ, might lead to *de novo* expression of HLA-DR on vaginal SMCs, thus enhancing their ability to perpetuate inflammation. This could be the underpinning mechanism of an organ-specific chronic, self-perpetuating inflammation in the human vagina, where infections easily initiate (Monin *et al.* 2020).

The GSM results from the complex interplay among decreased sex steroids levels and age-related systemic chronic inflammation ('Inflamm-aging') and changes in the immune response (Gameiro *et al.* 2010, Klein & Flanagan 2016, Simon *et al.* 2018). We here propose a novel immunoregulatory role played by androgens in the GSM and in other chronic inflammatory diseases affecting women's genital tract. The immunosuppressive role of androgens on key components of both innate and adaptive immunity has been consistently described in recent literature in pre-clinical and clinical models in males (Vignozzi *et al.* 2012*a*,*b*,*c*, Trigunaite *et al.* 2015). In particular, in vitro testosterone treatment of isolated mouse macrophages elicited a decrease in TLR4 expression and in sensitivity to a specific ligand (Rettew *et al.* 2008). *In vitro* treatment with androgens also resulted in inhibition of Th1 differentiation and less IFNγ production by the peripheral T cell population (Kissick *et al.* 2014). Noteworthy, in experimental animal model of metabolic syndrome-associated systemic low-grade inflammation, testosterone treatment exerted a potent anti-inflammatory action in several districts, including liver (Vignozzi *et al.* 2014), adipose tissue (Maneschi *et al.* 2012), bladder (Morelli *et al.* 2012) and prostate (Vignozzi *et al.* 2012*b*). Our data are consistent with this view and appear innovative in that, for the first time, expand the paradigm of the anti-inflammatory effects of androgens to females and specifically to the vagina.

In conclusion, our data suggest that vaginal SMCs have the ability to be involved in the immune response, acting as non-professional APCs, and that the activation of AR exerts an important anti-inflammatory activity by inhibiting their role in inducing and/or sustaining immune and inflammatory responses. Androgens represent a potential therapeutic strategy for the GSM, to be further investigated in clinical studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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