Antiinflammatory effect of androgen receptor activation in human benign prostatic hyperplasia cells

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Abstract

Progression of benign prostatic hyperplasia (BPH) involves chronic inflammation and immune dysregulation. Preclinical studies have demonstrated that prostate inflammation and tissue remodeling are exacerbated by hypogonadism and prevented by testosterone supplementation. We now investigated whether, in humans, hypogonadism was associated with more severe BPH inflammation and the *in vitro* effect of the selective androgen receptor agonist dihydrotestosterone (DHT) on cultures of stromal cells derived from BPH patients (hBPH). Histological analysis of inflammatory infiltrates in prostatectomy specimens from a cohort of BPH patients and correlation with serum testosterone level was performed. Even after adjusting for confounding factors, hypogonadism was associated with a fivefold increased risk of intraprostatic inflammation, which was also more severe than that observed in eugonadal BPH patients. Triggering hBPH cells by inflammatory stimuli (tumor necrosis factor α , lipopolysaccharide, or CD4⁺T cells) induced abundant

secretion of inflammatory/growth factors (interleukin 6 (IL6), IL8, and basic fibroblast growth factor (bFGF)). Co-culture of CD4⁺T cells with hBPH cells induced secretion of Th1 inducer (IL12), Th1-recruiting chemokine (interferon γ inducible protein 10, IP10), and Th2 (IL9)- and Th17 (IL17)-specific cytokines. Pretreatment with DHT inhibited NF- κ B activation and suppressed secretion of several inflammatory/growth factors, with the most pronounced effects on IL8, IL6, and bFGF. Reduced inflammatory cytokine production by testosterone cells, an increase in IL10, and a significant reduction of testosterone cells proliferation suggested that DHT exerted a broad antiinflammatory effect on testosterone cells. In conclusion, our data demonstrate that DHT exerts an immune regulatory role on human prostatic stromal cells, inhibiting their potential to actively induce and/or sustain autoimmune and inflammatory responses.

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Introduction

Benign prostatic hyperplasia (BPH) is a highly prevalent disorder, ranking among the ten most common diseases in aging men (Issa & Regan 2007). BPH is commonly viewed as a benign, progressive, growth of the prostate gland, which clinically translates into a spectrum of lower urinary tract symptoms (LUTSs), such as urgency, frequency, weak stream, nocturia, and incomplete bladder emptying.

Although primarily characterized by hyperproliferation of both stromal and epithelial cells, a wealth of recent epidemiological and histopathological studies have clearly

evidenced that chronic inflammation is a common finding in BPH (see Kramer *et al.* (2007), Fibbi *et al.* (2010b) and Schauer & Rowley (2011) for reviews). Data from two large-scale studies have indicated that chronic inflammatory infiltrates are associated with higher prostatic volume, a higher risk of BPH progression, and acute urinary retention (Roehrborn *et al.* 2007, Nickel *et al.* 2007, 2008). From a pathophysiological standpoint, these data suggest that chronic inflammation could have a causative effect on BPH/LUTS, rather than merely occurring in response to tissue remodeling. Accordingly, infiltration of chronically activated CD4⁺T lymphocytes and secretion of inflammatory cytokines

within the prostatic gland are considered a determinant factor in BPH pathogenesis (see Kramer *et al.* (2007), Fibbi *et al.* (2010b) and Schauer & Rowley (2011) for reviews).

Cytokines, chemokines, and growth factors are involved at each stage of BPH development and progression. In the initial phase, Th1 lymphocyte accumulation, producing interferon- γ (IFN- γ), and interleukin 2 (IL2), prevails and stimulates IL15 production by stromal cells, thus perpetuating the infiltration process. As in other chronic inflammatory immune disorders, a progressive switch from a Th1 response to a less polarized immune response is observed during the disease course (Rotondi *et al.* 2007). This shift is currently viewed as a counter-regulatory mechanism against inflammation, occurring when the Th1 response becomes dangerous for the host. Indeed, as testosterone lymphocytes progressively accumulate, IL4 and IL13 expression increases, suggesting a shift toward a Th0/Th2 immune response (Steiner *et al.* 2003a). There is also evidence that a loss of tolerance to self-antigens, associated with expansion of Th17 cells and IL17 overexpression, is crucial in BPH development (Steiner *et al.* 2003b). This is also supported by the observation that IL8 and IL6, key executors of stromal growth in BPH, are produced by prostatic stromal cells in response to IL17 (Penna *et al.* 2009), thus linking a self-perpetuating autoimmune response to altered tissue remodeling and hyperplastic growth (Ropiquet *et al.* 1999).

The causes for inflammation and immune dysregulation in the prostate remain subjects of debate. Potential causes include infectious agents, exposure to other environmental and dietary factors, hormonal and metabolic derangements, or a combination thereof. Animal models have provided a great deal of information about an association between metabolic diseases and LUT alterations (Azadzo *et al.* 1999, Kozlowski *et al.* 2001, Krajewska *et al.* 2008, Morelli *et al.* 2012, Vignozzi *et al.* 2012). Our laboratory has developed, over the last few years, an animal model of metabolic syndrome-associated hypogonadotropic hypogonadism by feeding adult male rabbits a high fat diet (HFD) for 12 weeks (Filippi *et al.* 2009, Vignozzi *et al.* 2011, 2012, Morelli *et al.* 2012). Interestingly, HFD-induced hypogonadism is associated with marked histological alterations of the prostate gland, characterized by inflammation coupled with stromal derangement and hypoxia, which are completely normalized by testosterone supplementation (Vignozzi *et al.* 2012). However, testosterone supplementation, in parallel with its beneficial effects on the prostate, ameliorates also several metabolic features in HFD-treated rabbits. Hence, a direct protective effect of testosterone on the prostate has not yet been conclusively demonstrated.

In the present work, we examined the antiinflammatory effect of the selective androgen receptor (AR) agonist dihydrotestosterone (DHT) on human prostate by performing a series of *in vitro* experiments using stromal cells isolated from BPH patients (hBPH). BPH cells have been previously described to act as antigen-presenting cells (APCs; Penna *et al.* 2009), thus indicating their potential role in inducing and

sustaining an autoimmune response within the prostatic gland. Histological analysis of inflammatory cell infiltrates in prostatectomy specimens from a cohort of BPH patients and their correlation with preoperative serum testosterone levels were also performed.

Materials and Methods

Collection of human prostate specimens

All patients underwent a routine prostatic evaluation, including digital rectal examination, prostatic transrectal ultrasound (TRUS), and measurement of serum total prostate-specific antigen (PSA) level, to exclude the presence of prostatic carcinoma. TRUS was also used to measure the adenoma volume, using the ellipsoidal formula. Suprapubic transvesical prostatectomy was performed under general or spinal anesthesia using a modified Freyer technique, comprising anterior bladder access, enucleation of the adenoma through a circular bladder neck incision, urethral section, and suturing of the prostatic groove.

Transurethral resection of prostate (TURP) was performed with resectoscopes and cutting loops, removing the hyperplastic prostatic tissue of the transition zone. The cutting of prostatic tissue and coagulation of blood vessels are achieved by using adaptable electrical current. Surgical specimens were collected by a sterile procedure and used for both histological examination and laboratory workup after informed consent.

Hypogonadism was defined according to different total testosterone thresholds (≤ 8 , ≤ 10.4 , and ≤ 12 nmol/l). When the different thresholds were applied 9.5, 21.4, and 38.1% of the population samples respectively satisfied the criteria. Severe hypogonadism was defined as total testosterone ≤ 8 nmol/l (230 ng/dl).

Pathological assessment of prostatic inflammatory infiltrates

A series of 42 patients undergoing open prostatectomy for BPH were analyzed by two independent pathologists (G N and R S), blinded to clinical findings. All surgical specimens were investigated for the presence of an inflammatory infiltrate, according to the standardized classification system of chronic prostatitis/chronic pelvic pain syndrome of the National Institutes of Health (NIH; Nickel *et al.* 2001). The following parameters were assessed: prevalent anatomical location (stromal, periglandular, and glandular), grade (mild, moderate, and severe), and extent (focal (10%), multifocal (10–50%), and diffuse ($> 50\%$)) of inflammatory infiltrates and presence/absence of glandular disruption. For the purposes of statistical analysis, an ‘inflammatory score’ (IS) combining all the above-mentioned histological parameters was defined. Five micron-thick sections were cut from formalin-fixed paraffin-embedded prostatic tissues for immunohistochemical analysis. A mouse monoclonal ready-to-use anti-CD45

antibody (Ab; clone 2B11 and PD7/26; Ventana Medical Systems, Inc., Tucson, AZ, USA) was employed as Ab.

All tissue sections were placed on the Ventana automated stainer BenchMark XT ICH system where they were deparaffinized, rehydrated, and processed for blocking the endogenous peroxidase and epitope retrieval. Following the Ventana staining procedure, the primary Ab was then placed on the tissue sections and incubated for 32 min at 37 °C, using the iVIEW DAB detection kit as the revelation system. After the staining run had been completed, the tissue sections were removed from the stainer, counterstained with Mayer's hematoxylin, dehydrated, and mounted in permanent mounting medium. The negative control was performed by substituting the primary Ab with a Ventana dispenser filled with nonimmune serum at the same concentration as the primary Ab. Known positive controls were used throughout. The control sections were treated in parallel with the samples and in the same run.

Human prostatic stromal cell cultures

Primary human prostatic smooth muscle cells (hBPH) were obtained and cultured as previously described (Crescioli *et al.* 2000, Fibbi *et al.* 2010a,b). Briefly, six different hBPH cell preparations were obtained from prostate tissues derived from six patients who underwent TURP or suprapubic adenomectomy for BPH after informed consent. Patients did not receive any pharmacological treatment in the 3 months preceding surgery. Surgical specimens were cut into small fragments and treated overnight with 2 mg/ml bacterial collagenase type IV (Sigma-Aldrich). Fragments were then extensively washed in PBS and cultured in a DMEM-F12 1:1 mixture supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in a fully humidified atmosphere of 95% air and 5% CO₂. Cells began to emerge within 1 week and were used within the tenth passage.

Cytokine and chemokine production by BPH cells

Basal secretion of cytokines and chemokines in not-irradiated BPH and the antiinflammatory effects of DHT were tested in a different set of experiments as follows: BPH cells were stimulated with tumor necrosis factor α (TNF α ; 10 ng/ml for 5 h) or lipopolysaccharide (LPS; 100 ng/ml for 48 h) in the presence or absence of DHT (30 nM) added 24 h before cell stimulation. Cell culture supernatants were analyzed with the bead-based multiplex assay (Bio-Rad). Experiments were performed in triplicate using four different BPH cell preparations.

Generation of CD4⁺ T cell clones from peripheral blood of healthy subjects

To generate T-cell clones, peripheral blood mononuclear cells (PBMCs) from normal subjects were seeded under limiting

dilution conditions (0.3 cells/well) in round-bottomed microwell plates containing 10⁵ irradiated (9000 rads) allogeneic PBMCs (as feeder cells) and Phytohemagglutinin (PHA) (1% vol/vol; Gibco Laboratories) in a final volume of 0.2 ml of RPMI-1640 supplemented with 2 mM L-glutamine, 2 × 10⁻⁵ M 2-mercaptoethanol, IL2 (50 U/ml; Eurocetus, Milano, Italy), and 10% FCS (HyClone Laboratories, Inc., Logan, UT, USA), as we reported elsewhere (Piccinni *et al.* 1995). Growing microcultures were then supplemented, at weekly intervals, with IL2 (50 U/ml) and irradiated feeder cells. The phenotype distribution of T-cell clones was assessed by flow cytometric analysis, using anti-CD4 and anti-CD8 MABs (Becton Dickinson, Mountain View, CA, USA). For co-cultures with hBPH cells, CD3⁺ CD4⁺ T cell clones showing a Th0 profile were selected, thus producing IL4, IL5, IL13, and IFN- γ as measured by multiplex bead-based assay (Bio-Rad).

Determination of cytokine and chemokine concentrations

The quantitative determination of cytokines was performed with a bead-based multiplex immunoassay, as previously described (Lédée *et al.* 2008). Briefly a bead-based multiplex immunoassay (Bio-Rad) and a Bioplex 200 system (Bio-Rad; Luminex Map Technology) were used to measure simultaneously in cell culture supernatants the concentrations of the following cytokines and chemokines: IL1 β , IL1Ra, IL2, IL4, IL5, IL6, IL8, IL9, IL10, IL12, IL13, IL15, IL17A, IFN- γ , TNF α , G-CSF, GM-CSF, VEGF, PDGF, basic fibroblast growth factor (bFGF) interferon γ /inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), RANTES, eotaxin, macrophage inflammatory protein-1 α (MIP-1 α), and MIP-1 β . In brief, 50 µl of each supernatant were added to 50 µl of Ab-conjugated beads directed against the analytes listed above (Bio-Rad) in a 96-well filter plate (Bio-Rad). After a 30-min incubation, the plate was washed and 25 µl of biotinylated anticytokine Ab solution was added to each well before another 30-min incubation. The plate was then washed and 50 µl of streptavidin-conjugated phycoerythrin (PE) were added to each well. After a final wash, each well was resuspended with 125 µl of assay buffer (Bio-Rad) and analyzed with the Bioplex 200 system (Bio-Rad). 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 calculated thanks to the software of the Bioplex. The assay sensitivity for all proteins tested was <1 pg/ml.

Proliferation of T-cell clones stimulated by immobilized anti-CD3 Abs in the presence of irradiated hBPH cells

BPH cells pretreated or not with 30 nM DHT for 24 h were then irradiated (9000 rads) and co-cultured with CD3⁺ CD4⁺ Th0-type T-cell clones for 48 h. In brief, 10⁵ T-cell blasts obtained from T-cell clones in 0.2 ml RPMI-1640

medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-mercaptoethanol and 10% FCS (Hyclone Laboratories, Inc.) were stimulated in 96 U-bottomed plates with immobilized anti-CD3 antibodies (Ortho Pharmaceuticals, Raritan, NJ, USA) in the absence or presence of 3×10^4 irradiated (9000 rads) hBPH cells for 48 h. After a 16-h pulse with 0.5 μ Ci 3 H-TdR (Amersham International), cultures were harvested and radioactivity measured by liquid scintillation.

Induction of cytokine and chemokine production by CD4⁺ Th0-type T-cell clones in response to irradiated hBPH

Irradiated (9000 rads) BPH cells, pretreated or not with 30 nM DHT for 24 h, were co-cultured in 0.2 ml of complete medium in 96 U-bottomed plates with CD3⁺ CD4⁺ Th0-type T-cell clones for 48 h, as described above. After 48 h, supernatants were collected and stored in aliquots at -80°C until used. The experiments were performed in triplicate using six different BPH cell preparations. Each T-cell clone was separately co-cultured with each BPH cell preparation.

The modulation of each T-cell clone was verified by cultures of immobilized anti-CD3 CD4⁺ T cell clones cultured in the presence of IL12 (5 ng/ml), which is a well-known inducer of IFN- γ production. If the T-cell clones did not show an increased production of IFN- γ , the T-cell clones were not modulated and therefore were excluded from the statistical analysis of our results. A total of 15 IL12-responsive T-cell clones were analyzed statistically.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from hBPH samples using RNeasy kit (Qiagen), according to the manufacturer's instructions, and cDNA synthesis was carried out using the RT kit purchased from Applied Biosystems. Quantitative RT-PCR (qRT-PCR) was performed by TaqMan Real-Time PCR Master Mix (Applied Biosystems) with the following thermal cycler conditions: 40 cycles at 95°C for 15 s and 60°C for 1 min. Primers and probe for human cyclooxygenase-2 (COX2 or PTGS2 according to the HUGO Database) was purchased from Applied Biosystems (assay ID: Hs00153133_m1). Amplification and detection were performed with MyiQTM 2 Two-Color Real-Time PCR Detection System (Bio-Rad). The expression of 18S rRNA subunit, chosen as reference gene, was quantified with a predeveloped assay (Applied Biosystems) and used for normalization and relative quantitation of the target gene. Data analysis was based on the comparative threshold cycle (C_t) method according to the manufacturer's instructions (Applied Biosystems), as previously described (Zhang *et al.* 2005).

Immunofluorescence microscopy

Cells (10^4) were seeded on glass coverslips in growth medium. After 24 h of serum starvation, cells were incubated with

DHT (30 nM) or left untreated for 24 h, then were stimulated or not with TNF α (10 ng/ml) or with LPS (100 ng/ml) for 5 h. Cells cultured in phenol red and serum-free medium were used as control. Immunostaining was performed as previously described (Penna *et al.* 2009) using primary Abs against NF- κ B p65 (1:100) followed by Alexa Fluor conjugated secondary Abs (1:200). Slides were then treated as reported elsewhere (Penna *et al.* 2009) and examined with a phase contrast microscope (Nikon Microphot-FX microscope, Nikon, Tokyo, Japan). Experiments were performed three times with different cell preparations.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Comparisons of means were performed with one-way ANOVA followed by unpaired two-sided Student's *t*-tests. $P < 0.05$ was taken as significant. Correlations were assessed using Spearman's method. Stepwise multiple linear or logistic regressions were applied for multivariate analysis, whenever appropriate. Relative risk and 95% confidence interval were calculated for association of categorical parameters.

All statistical analysis was performed on SPSS (SPSS, Inc., Chicago, IL, USA) for Windows 17.0. Values of half-maximal response inhibitory concentration (IC_{50}) as well as maximal inhibitory (I_{max}) effect were calculated by using the ALLFIT program (De Lean *et al.* 1978).

Results

Association between inflammation and testosterone levels

Histological features of inflammatory infiltrate specimens derived from 42 BPH patients undergoing simple prostatectomy were analyzed blindly and scored according to a previously validated protocol (Nickel *et al.* 2001). The demographic and clinical characteristics of the patients analyzed are summarized in Table 1. Histopathological examination of BPH specimens demonstrated the presence of prostatic inflammation in all cases. The IS was higher in severe hypogonadal (testosterone ≤ 8 nM) than eugonadal (testosterone > 8 nM) patients ($P < 0.01$, Fig. 1 panel a). Accordingly, severe hypogonadism increased the risk of prostate inflammation by a factor of five, even after adjusting for age and body mass index (BMI; HR = 5.7 (1.1–29.4), $P < 0.05$). In an age- and BMI-adjusted model, among the different factors composing the IS, the inflammatory infiltrate grade showed a significant, negative association with testosterone levels (adjusted $R = -0.35$, $P = 0.03$, Fig. 1 panel a, inset, and panel b). When patients were stratified according to current treatments, including 5 α -reductase inhibitors, no differences were found for inflammatory parameters (not shown).

Figure 1 shows histological patterns of prostatic inflammatory infiltrates in two representative patients (eugonadal:

Table 1 Characteristics of the patients analyzed

	Values
Clinical features	
Age (years)	70.0±7.4 (51.0–83.0)
BMI	26.0±3.0 (21.0–37.4)
Metabolic syndrome features	
Waist circumference (cm)	98.7±8.3 (87.0–131.0)
DBP (mmHg)	74.3±6.4 (60–90)
SBP (mmHg)	131.3±12.8 (110.0–165.0)
HDL (mg/dl)	48.4±5.4 (39.0–61.0)
Triglycerides (mg/dl)	112.9±28.3 (62.0–175.0)
Glycemia (mg/dl)	100.5±24.3 (70.0–207.0)
Hormonal features	
Testosterone (nM)	14.8±5.7 (5.4–31.5)
FSH (U/l)	8.7±7.2 (1.8–28.0)
LH (U/l)	5.6±3.3 (1.4–13.3)
Pathology	
Prostate sample weight (mg)	76.4±15.5 (40.0–110.0)

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein. Data are expressed as mean±s.d.; the range is indicated in brackets.

Fig. 1 panels c and d, and hypogonadal: Fig. 1 panels e and f). In the eugonadal patient, a stromal scattering of individual inflammatory cells (typically lymphocytes) was documented (Fig. 1 panels c and d). By contrast, the prostate specimen from the hypogonadal patient displayed a marked stromal and periglandular inflammation, with intraepithelial and luminal inflammatory infiltrates causing glandular disruption (Fig. 1 panels e and f). CD45-positive cells were extensively present in both interductal stroma and intertwined within the epithelial glands in the hypogonadal patient (Fig. 1 panel f), while only scanty CD45-positive cells were present in the prostate specimen from the eugonadal subject (Fig. 1 panel d).

DHT treatment inhibits basal secretion of proinflammatory cytokines/chemokines and growth factors by human BPH cells

To investigate whether AR is involved in mediating the antiinflammatory effect of testosterone, the potent and selective AR ligand DHT was used. Experiments were performed in well-established stromal cell cultures isolated from BPH samples (*n*=6 different preparations). We first measured cytokines, chemokines, and growth factors detected in culture supernatants of BPH cells (Fig. 2).

Figure 1 Histological features of intraprostatic inflammatory infiltrates in hypogonadal and eugonadal BPH subjects. (Panel a) Total inflammation score and grade (inset) of prostatic inflammation in hypogonadal (total testosterone ≤8 nM) and eugonadal (total testosterone >8 nM) BPH subjects. (Panel b) Testosterone level as a function of intraprostatic inflammatory grade score. Inset indicates the age- and BMI-adjusted data. (Panels c–f) Frozen prostate samples from a representative eugonadal (panels c and d: total testosterone=21.3 nM) and hypogonadal (panels e and f: total testosterone=5.4 nM) patients were stained with H&E (panels c and e) or with a specific Ab anti-pan leukocyte marker (CD45, panels d and f). Original magnification, 10×. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0142>.

Figure 2 Cytokines, chemokines, and growth factor levels in hBPH cell cultures. Levels (pg/ml) of the indicated cytokines, chemokines, and growth factors were determined in culture supernatants by bead-based multiplex immunoassay. Data are expressed as mean $\pm$ S.D. of basal secretion in six different preparations of hBPH cells obtained from prostate tissues of six different BPH patients.

VEGF, eotaxin, MCP-1 (CCL2), IL6, and IL8 were the most abundantly secreted products (>1000 pg/ml). Strikingly, after DHT treatment of BPH cells (30 nM for 24 h), a clear overall reduction in the secretion of inflammatory products was observed, with significant decreases seen for IL6, IL8, MCP-1, bFGF, IL7, IL9, INF- γ , IP-10 (CXCL10), IL12p75, and G-CSF (Fig. 3).

DHT inhibits TNF α -induced secretion of proinflammatory cytokines/chemokines and growth factors in hBPH cells

The secretion of proinflammatory mediators and the effect of DHT (30 nM for 24 h) were also studied in BPH cells after TNF α priming (10 ng/ml for 5 h). TNF α upregulated basal secretion of IL8, IL6, IL9, IL12p75, IP-10, MCP-1, and bFGF (Fig. 4), with no effect on the other 20 cytokines and chemokines measured. Pretreatment with DHT significantly blunted TNF α -induced secretion of IL8, IL6, IL9, IL12p75, IP-10, MCP-1, and bFGF (Fig. 4 panels a, b, c, d, e, f and g). The effect of DHT was dose-dependent (shared $IC_{50} = 4.56 \pm 1.4 \times 10^{-11}$ M; Fig. 4 panel h), although with different I_{max} effects (Fig. 4 panel i).

DHT inhibits NF- κ B p65 activation in TNF α -stimulated BPH cells

To better characterize the effect of DHT (30 nM) on the inflammatory response of TNF α -stimulated BPH cells, nuclear translocation of NF- κ B p65 was studied. As assessed by immunofluorescence, in untreated BPH cells NF- κ B p65 was totally retained in the cytoplasm (Fig. 5 panel e). TNF α induced a complete translocation of NF- κ B p65 to the nucleus, which was inhibited ($56.9 \pm 3.8\%$; $P < 0.0001$ vs TNF α), but not normalized ($P < 0.001$ vs untreated BPH cells) by DHT (Fig. 5 panel e). Representative images are shown in Fig. 5 (panels a, b, c, and d). Accordingly, TNF α -induced mRNA expression of the NF- κ B p65 target gene *COX2* (*PTGS2*; $P < 0.01$, Fig. 5 panel f) was significantly reduced by pretreatment with DHT ($P < 0.05$).

DHT inhibits LPS-induced secretion of proinflammatory cytokines/chemokines and growth factors and NF- κ B translocation and signaling in hBPH cells

We next tested the capacity of DHT to reduce the secretion of proinflammatory cytokines/chemokines and growth factor secretion in LPS-primed BPH cells. LPS treatment (100 ng/ml for 48 h) induced a significantly increased production of IL1 β , IL1RA, G-CSF, TNF α , eotaxin, IFN- γ , IP-10, IL4, IL7, IL6, and IL8. Among these cytokines, only IL8, IL6, IL7, IP-10, TNF α , and IL1RA were significantly reduced by pretreatment with DHT ($P < 0.05$; Fig. 6 panels a, b, c, d, e and f). Immunolocalization analysis demonstrated that LPS induced a 50% translocation of NF- κ B p65 to the nucleus, which was significantly inhibited ($15 \pm 3.6\%$; $P < 0.0001$ vs LPS), but not normalized ($P < 0.001$ vs untreated BPH cells) by DHT preincubation

Figure 3 Effect of DHT treatment on cytokine/chemokine/growth factor secretion by BPH cells. hBPH cells were cultured for 24 h with serum-free medium alone (untreated) or DHT (30 nM). Culture supernatants were analyzed with a bead-based multiplex assay. Experiments were performed in triplicate using four different hBPH cell preparations. Data are expressed as percentage of variation vs untreated. * $P < 0.05$ vs untreated.

(Fig. 6 panel g). As previously observed in TNF α -stimulated BPH cells, LPS-induced mRNA expression of COX2 was also significantly reduced by DHT (Fig. 6 panel h).

Co-culture of BPH cells and activated CD4⁺T cell clones enhances production of proinflammatory cytokines/chemokines and growth factors

As hBPH cells can act as APCs (Penna *et al.* 2009), we evaluated in co-culture experiments the capacity of hBPH cells to modulate cytokine production by T-cells (Fig. 7). We found

that addition of hBPH cells to activated CD4⁺T cell clones resulted in significantly increased levels of several proinflammatory mediators, including IL1 β , IL15, TNF α , eotaxin, G-CSF, MCP-1, and MIP-1 α (CCL3). IL1RA production was also significantly enhanced. A significant increase of the Th1-inducer IL12 and Th1-type chemokine IFN- γ -IP-10 was also observed, although IFN- γ and IL2 production were not significantly modulated. Th2-type cytokines were also differentially modulated, with IL9 significantly increased and IL13 significantly decreased, whereas IL4 and IL5 were not significantly affected. By contrast, the T-cell growth factor IL7

Figure 4 DHT inhibits TNF α -induced secretion of cytokines, chemokines, and growth factors by hBPH cells. hBPH cells were stimulated with TNF α (10 ng/ml for 5 h) with or without preincubation with DHT (30 nM for 24 h). (Panels a, b, c, d, e, f and g). Culture supernatants were analyzed for production of the indicated cytokines, chemokines, and growth factors. The data represent the mean $\pm$ S.E.M. of four independent experiments performed in triplicate and are reported as percentage of untreated cells (control). * P <0.05, ** P <0.01 vs control; $^{\circ}$ P <0.05, $^{\circ\circ}$ P <0.01 vs TNF α . (Panel h) Inhibitory effect of increasing concentrations of DHT (1 pM–100 nM) on TNF α -induced cytokine, chemokine, or growth factor secretion by hBPH cells. Ordinate: secretion of the indicated cytokines, chemokines, and growth factors induced by TNF α (10 ng/ml for 5 h), expressed as percentage of the effect of TNF α alone. Abscissa: molar concentrations of DHT. The data represent the mean $\pm$ S.E.M. of four independent experiments performed in triplicate. The relative half-maximal response IC_{50} value is reported in the text, while relative I_{max} effects are reported in panel i.

Figure 5 DHT inhibits NF-κB p65 nuclear translocation in hBPH cells. hBPH cells were stimulated with TNFα (10 ng/ml for 5 h) with or without preincubation with DHT (30 nM for 24 h). After washing, hBPH cells were fixed and stained with anti-NF-κB p65 Ab. Cells were analyzed by fluorescence microscopy. Images of panels a, b, c and d are representative from one of three experiments performed (magnification 10×). (Panel e) Bar graph shows the number of cells with nuclear localization of NF-κB expressed in percentage of total cells. The data represent the mean ± s.e.m. of three independent experiments performed in triplicate. (Panel f) mRNA expression of COX2 was evaluated using qRT-PCR in hBPH cells untreated (control) and TNFα primed, with or without preincubation with DHT (30 nM for 24 h). Data were calculated according to comparative C_t method using 18S rRNA subunit as the reference gene for normalization. Results are expressed as percentage of control and are reported as mean ± s.e.m. of three independent experiments performed in triplicate. * $P < 0.001$ vs control; ** $P < 0.0001$ vs TNFα. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0142>.

and the Th17-specific cytokine IL17 were both significantly increased. In keeping with the overall proinflammatory effects of hBPH cells on cytokine production by T-cells, the antiinflammatory cytokine IL10 was significantly inhibited. Interestingly, a series of factors promoting BPH cell growth, such as IL8, IL6, bFGF, VEGF, and PDGFBB, were markedly enhanced by co-cultures.

Co-culture of DHT-pretreated BPH cells and activated CD4⁺T cell clones inhibit secretion of proinflammatory cytokine/chemokines and growth factors

When activated CD4⁺T cell clones were cultured with DHT-primed (30 nM for 24 h) hBPH cells, secretion of several proinflammatory factors was significantly affected (Fig. 8). In general, production of proinflammatory mediators was blunted, with a significant reduction observed for MCP-1, MIP-1α, and MIP-1β. Cytokine production by effector T-cells was consistently reduced, with significant

decreases seen for IL2, IL4, IL13, and IL17. Also production of the Th1-recruiting chemokine IP-10 was significantly decreased. By contrast, IL10 was significantly increased, consistent with the antiinflammatory activity of DHT. In line with the antiinflammatory properties displayed by DHT, factors promoting hBPH cell growth were mostly decreased, with significant effects seen for IL8, IL6, and bFGF, whereas VEGF secretion was increased.

DHT-pretreated BPH cells inhibit proliferation of activated CD4⁺T cells

Proliferation of CD4⁺T cell clones, as measured by [³H]thymidine incorporation, was significantly increased by activation with anti-CD3 Ab ($P < 0.05$), while it was not affected by the addition of irradiated hBPH cells (Fig. 9). However, when hBPH cells were pretreated with DHT (30 nM for 24 h), a significant inhibition of T-cell proliferation was observed ($P < 0.01$; Fig. 9).

Figure 6 DHT inhibits LPS-induced inflammatory response in BPH cells. BPH cells were stimulated with LPS (100 ng/ml, for 48 h) with or without preincubation with DHT (30 nM for 24 h). Cell culture supernatants were analyzed for production of the indicated cytokines, chemokines, and growth factors (panels a, b, c, d, e and f). The data represent the mean $\pm$ S.E.M. of four independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs control; ° $P < 0.05$ vs LPS. (Panel g) NF- κ B p65 nuclear translocation in BPH cells stimulated by LPS (100 ng/ml, for 5 h), expressed in percentage of total cells. The data represent the mean $\pm$ S.E.M. of four independent experiments performed in triplicate. (* $P < 0.001$ vs untreated hBPH cells; ° $P < 0.0001$ vs LPS). (Panel h) mRNA expression of COX2 was evaluated using qRT-PCR in hBPH cells untreated (control) and LPS-primed pretreated or not with DHT (30 nM, for 24 h). Data were calculated according to the comparative C_t method using 18S rRNA subunit as the reference gene for normalization. Results are expressed in percentage of control and are reported as mean $\pm$ S.E.M. of three independent experiments performed in triplicate. * $P < 0.001$ vs control, ° $P < 0.0001$ vs LPS.

Discussion

Results in this study demonstrate, for the first time, that AR activation exerts a direct antiinflammatory effect on human stromal prostate cells, thus inhibiting their potential to induce and sustain autoimmune and inflammatory responses.

Chronic intraprostatic inflammation and subsequent chronic tissue remodeling are determinant factors in the development and progression of prostatic diseases, including BPH (see Kramer *et al.* (2007), Fibbi *et al.* (2010b) and Schauer & Rowley (2011) for reviews). A possible hormonal basis for prostate inflammation is suggested by preclinical studies in animal models, demonstrating that hypogonadism induced surgically (Robinette 1988, Desai *et al.* 2004, Quintar *et al.* 2006, Meng *et al.* 2011) or by HFD administration (Vignozzi *et al.* 2012) exacerbate prostate inflammation and that exogenous testosterone can counteract this effect. In particular, we have shown in a rabbit model that testosterone supplementation can prevent HFD-induced

prostatic alterations, including inflammation, tissue remodeling, and hypoxia (Vignozzi *et al.* 2012).

In the present study, we sought to provide evidence supporting the hypothesis that low androgen levels could enhance inflammatory responses in the human prostate. Hence, we first retrospectively examined the histological features of inflammatory infiltrates in prostatectomy specimens derived from a cohort of BPH patients. Even after adjusting for confounding factors, hypogonadism was associated with a fivefold increased risk of intraprostatic inflammation, which was also more severe than that observed in eugonadal BPH patients. Although it is historically assumed that high testosterone induces prostate overgrowth, most observational studies failed to find correlations between circulating testosterone levels and BPH: in fact, no clear correlation with serum PSA or prostate volume across the normal testosterone range has been shown (Liu *et al.* 2007). In addition, the notion that intraprostatic 5 α -reductase activity, which is responsible for converting the bulk of testosterone

Figure 7 Secretion of cytokines, chemokines, and growth factor levels by hBPH cell/CD4⁺ T cell co-cultures. Concentration (pg/ml) of the indicated cytokines, chemokines, and growth factors were determined in the supernatants by bead-based multiplex immunoassay. Data are expressed as log (percentage of variation vs activated CD4⁺ T cells alone) and reported as mean $\pm$ S.E.M. of three individual experiments. * $P < 0.05$, ** $P < 0.001$ vs CD4⁺ T cells.

into DHT, is altered in the BPH tissues is contentious (Isaacs *et al.* 1983, Bartsch *et al.* 1990). However, it could be hypothesized that intraprostatic level of DHT could be more important than the level of serum testosterone for the growth of the prostate (Isaacs *et al.* 1983), thus explaining this lack of association between serum testosterone level and prostate overgrowth. In contrast, some uncontrolled studies have reported a gradual improvement in the International Prostate Symptom Score (IPSS) following long-term testosterone therapy in men with hypogonadism and/or metabolic syndrome (MetS) (see review in Buvat *et al.* (2010)). A small randomized controlled trial with testosterone enanthate in 23 men with BPH tends to support these findings, with a significant decrease in the IPSS score, maximum flow rate, and voided volume in the testosterone group but not in the 23 untreated controls (Shigehara *et al.* 2011). However, in the present sample we did not find any difference in inflammation in BPH specimens from subjects treated or not with 5 α -reductase inhibitors.

To better investigate whether androgens could directly suppress prostate inflammatory responses, we performed *in vitro* studies in human prostatic stromal cell cultures using the potent and selective AR ligand DHT. We have previously demonstrated the ability of hBPH cells to function as APCs, and to actively contribute to the organ-specific inflammatory process (Penna *et al.* 2009). Herein, we confirm that hBPH stromal cells secrete several proinflammatory and growth factors. Among them, IL8, IL6, MCP-1, VEGF, and eotaxin were the most abundantly secreted. A similar pattern of cytokine and chemokine secretion was previously described in seminal plasma of patients affected by BPH (Penna *et al.* 2007). In particular, seminal plasma IL8 was identified as a reliable surrogate marker

of prostatic inflammatory diseases and BPH, thanks to the fact that its level positively correlated with serum PSA, with prostatitis symptom score (Penna *et al.* 2007), and with ultrasonographic features of prostate inflammation (Lotti *et al.* 2011). The present data show secretion by stromal hBPH cells of many cytokines, chemokines, and growth factors induced by inflammatory stimuli, including TNF α , LPS, or activated CD4⁺ T cells. Secretion of IL8 and IL6 was markedly upregulated, as was production of bFGF, suggesting that an activated immune system might sustain prostate overgrowth. IL8, a primary cytokine in the recruitment of neutrophils into the inflammatory sites, showed also a potent proliferative action in prostate. Indeed, IL8 induced stromal BPH cell overgrowth by directly promoting the fibroblast-to-myofibroblast trans-differentiation and by indirectly stimulating secretion of bFGF, which acts as a potent prostatic growth factor. Similar to IL8, IL6 directly promotes stromal cell proliferation in an autocrine manner (see Fibbi *et al.* (2010b) for a review). The increased production of several chemokines, including IP-10, IL8, MCP-1, MIP-1 α , and MIP-1 β in co-cultures of BPH cells with activated CD4⁺ T cell clones suggested the capacity of BPH cells to recruit Th1 cells and other immune cells into the inflamed prostate. A concomitant increase of IL1 β , IL1RA, and IL15, cytokines described to be highly upregulated in BPH (see Kramer *et al.* (2007) for a review) has also been observed.

Our data also demonstrate that hBPH cells can influence CD4⁺ T cell activation, modulating their phenotype. When activated CD4⁺ T cell clones were co-cultured with hBPH cells, a significant increase of a Th1 inducer (IL12), a Th1-recruiting chemokine (IP-10), a Th2-type cytokine (IL9), and a Th17-specific cytokine (IL17) was observed. In addition, IL7, a crucial cytokine for survival and expansion of Th17 cells

Figure 8 Secretion of cytokines, chemokines, and growth factor levels by DHT-treated hBPH cell/CD4⁺T cell co-cultures. Concentrations (pg/ml) of the indicated cytokines, chemokines, and growth factors were determined in the supernatants by bead-based multiplex immunoassay. Data are expressed as percentage of variation vs untreated hBPH cell/CD4⁺T cell co-cultures and reported as mean $\pm$ S.E.M. of three individual experiments. * $P < 0.05$, ** $P < 0.001$ vs untreated hBPH/CD4⁺T cell co-cultures.

in autoimmune disease models (Kanai *et al.* 2009, Liu *et al.* 2010) was also increased. Concomitantly, a marked decrease of the antiinflammatory cytokine IL10 was also observed.

The most striking finding of the present study is that activation of AR by DHT markedly suppresses the inflammatory response and secretion of growth factors in hBPH cells, thus suggesting that stromal cell AR plays an important role in maintaining adult prostate homeostasis. This is in agreement with the observation that the selective ablation of AR in mouse prostate stromal cells causes a diffuse stromal hyperplasia mostly characterized by infiltration of leukocytes (neutrophils and monocytes) in adult prostate gland (Welsh *et al.* 2011).

The prevalent inhibitory effect of DHT was observed in IL8 and bFGF secretion, both in basal and stimulated conditions. Interestingly, this antiinflammatory effect of DHT was exerted at very low concentrations (10^{-11} mol/l), roughly corresponding to the K_d of DHT for the AR in hBPH cells (Crescioli *et al.* 2003).

In the present study we also demonstrated that DHT-primed hBPH cells co-cultures with CD4⁺T cell clones caused a reduction in cytokines produced by effector T-cells and an increase in IL10 production. This suggests that DHT could play a broad antiinflammatory role on CD4⁺T helper cells. Moreover, DHT inhibited the production of IL2, T-cell growth and differentiation (see Liao *et al.* (2011)), thus explained the reduced proliferation of CD4⁺T cell clones in culture with DHT-primed hBPH cells.

The mechanisms by which DHT exerts its antiinflammatory effects are not completely understood. We demonstrate that DHT inhibits NF- κ B activation, a master transcription factor in inflammation, as evidenced by its reduced nuclear translocation and by the decreased expression of COX2. A similar antiinflammatory effect has been described also in human endothelial cells, where DHT or testosterone

Figure 9 Proliferation of activated CD4⁺T cell clones in the presence of irradiated hBPH cells pretreated or not with DHT. Irradiated (9000 rads) hBPH cells, pretreated or not with 30 nM DHT for 24 h, were co-cultured with CD4⁺T cell clones for 48 h, then proliferation was measured by [³H]thymidine incorporation. Data represent the mean $\pm$ S.E.M. of three independent experiments * $P < 0.05$ vs not-activated CD4⁺T cell clones alone; ** $P < 0.01$ vs activated CD4⁺T cell clones; ° $P < 0.05$ vs activated CD4⁺T cell clones co-cultured with hBPH cells.

decreased TNF α -induced inflammatory response through the inhibition of NF- κ B signaling pathway (Hatakeyama *et al.* 2002, Norata *et al.* 2006).

Alternative, direct antiinflammatory mechanisms are also plausible, as suggested by a specific androgen response element present in genes related to inflammatory and proliferative response, such as IL6, described in rat prostate epithelial cells (Asirvatham *et al.* 2006).

In conclusion, our data demonstrate that DHT exerts an immune regulatory role on human prostatic stromal cells, inhibiting their potential to actively induce and/or sustain autoimmune and inflammatory responses. The prostate is an immunocompetent organ, not only because it is populated by resident inflammatory cells, including T- and B-lymphocytes, macrophages, and mast cells (De Marzo *et al.* 2007, Fibbi *et al.* 2010a,b), but also because stromal prostatic cells can secrete several proinflammatory cytokines and are able to recruit and activate CD4⁺ cells into the inflamed prostate. Under most conditions, this immune competence of the prostate would be beneficial to the host. However, in some situations, an immune response toward a Th1/Th17 cytokine profile might lead to the development of chronic immune-mediated tissue destruction and fibromyomatous growth, as observed in the pathogenesis of BPH. Interestingly, our data indicate that AR signaling might restrain, rather than facilitate, prostate inflammation.

Thus, DHT should be considered more a friend than a foe of prostate cells, consistent with the observation that prostate glands from hypogonadal subjects are more inflamed than those from eugonadal ones. Interventional studies aimed at evaluating the antiinflammatory effects of testosterone-replacement therapy in hypogonadal subjects with BPH are therefore warranted.

Declaration of interest

L V, I C, A M, P C, S F, M G, M C, F L, L L, M-P P, G N, and R S have no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported and have nothing to declare. M M is a scientific consultant for Bayer Pharma AG, Germany, and Eli-Lilly Indianapolis, Indiana. L A is an employee of Intercept Pharmaceuticals 18 Desbrosses Street, New York, NY 10013, USA.

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