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ORIGINAL ARTICLE

Prostate Disease

Combined treatment with dihydrotestosterone and lipopolysaccharide modulates prostate homeostasis by upregulating TNF-α from M1 macrophages and promotes proliferation of prostate stromal cells

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Androgens and chronic inflammation, which play essential roles in the development of benign prostatic hyperplasia (BPH), are considered to be important factors in disorders of prostate homeostasis. These two factors may lead to pathological hyperplasia in the prostate transition zone of patients with BPH. However, few studies have examined the mechanism of how dihydrotestosterone (DHT) affects chronic inflammation in prostate tissue during the progression of BPH. This study examined the performance of DHT in lipopolysaccharide-treated M1 macrophages and the subsequent effects on the proliferation of prostate stromal and epithelial cells. We found that DHT increased secretion of the pro-inflammatory factor tumor necrosis factor (TNF)-α from M1 macrophages differentiated from THP-1 cells. The supernatant of M1 macrophages promoted the proliferation of WPMY-1 prostate stromal cells by upregulating B-cell lymphoma-extra large (Bcl-xL) and cellular Myc (c-Myc) levels by activating TNF-α-mediated nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. Moreover, this supernatant increased the expression of androgen receptor in WPMY-1 cells, which was TNF-α-independent. Additionally, TNF-α protein expression was significantly higher in patients with BPH and a large prostate volume than that in those with a small prostate volume. Further analysis showed that higher serum testosterone combined with prostate-specific androgen concentrations was related to TNF-α expression. This study suggests that DHT modulates the inflammatory environment of BPH by increasing TNF-α expression from lipopolysaccharide-treated M1 macrophages and promotes the proliferation of prostate stromal cells. Targeting TNF-α, but not DHT, may be a promising strategy for patients with BPH.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a common disease in older men,[1] and its main pathological characters include proliferative hypertrophy with nodule formation in the transition zone in the prostate. The basic mechanisms of BPH are still unclear. However, androgens and upregulation of chronic inflammation are considered to be crucial factors promoting homeostasis disorders in the prostate, thereby playing an essential role in the occurrence and progression of BPH.[2,3]

In the prostate, dihydrotestosterone (DHT), which is derived from testosterone (T), is vital in gland development, function, and progression of pathological processes.[4] After DHT binds with androgen receptor (AR) in the cells of prostate tissue, it directly stimulates the expression of proliferation-related genes and accelerates cell division.[5] Age-related changes in the homeostasis of the prostate may modulate the microenvironment of the prostate, which disturbs the balance between tissue proliferation and apoptosis, leading to prostatic

hypertrophy. Inhibiting the synthesis of DHT has been shown to be an effective treatment for BPH.[6] Although taking a 5α-reductase inhibitor (5ARI) is currently the most common treatment for BPH, individual differences may lead to large differences in the therapeutic effects of 5ARIs. Moreover, serious adverse effects, such as irreversible or even persistent sexual, neurological, and mental side effects, can be caused by 5ARIs.[7] An in-depth study of the mechanism of how DHT affects the occurrence and development of BPH may help to find better treatments for patients with BPH.

The prostate is an immune-competent organ, which is characterized by a complicated intraglandular immune system.[8,9] Various immunocytes, including T cells and macrophages, are commonly observed in BPH tissue.[10] Antigen presentation and inflammatory factors secreted from macrophages are closely related to the progression of BPH. M1 macrophages show a pro-inflammatory phenotype and secrete pro-inflammatory cytokines in the initial and sustained stage of inflammation.[11]

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Androgens and inflammation can interact in BPH. Some researchers have found that androgens inhibit inflammation. After analyzing Prostate Cancer Prevention Trial (PCPT) data, Murtola *et al.*[12] found that the incidence of prostate inflammation was significantly increased after taking finasteride in the benign and malignant groups.[12] However, there was no significant difference in prostate-specific antigen (PSA) concentrations between the groups. Additionally, this increase in inflammation was accompanied by a decrease in the prostate volume (PV). Therefore, whether upregulated inflammation affects the progression of BPH after finasteride administration and which types of inflammatory factors play a role in the progress of BPH are unclear. Our previous study reviewed the role of androgens and inflammation in BPH and showed that the interaction between DHT and inflammation could play a vital role in the progression of BPH.[13] Nonetheless, the mechanism of the interaction between DHT and inflammation in patients with BPH has rarely been systematically studied.

This study aimed to investigate the combined effect of DHT and inflammation on BPH by the cell model of THP-1 cells after co-stimulation of DHT and lipopolysaccharide (LPS) and its consequential effects on the prostate.

PATIENTS AND METHODS

Prostate tissue specimens

Patients whose tissue specimens were acquired from holmium laser enucleation of the prostate (HoLEP) with the diagnosis of BPH at Jing'an District Central Hospital affiliated to Fudan University (Shanghai, China) between January 2016 and December 2019 (*n* = 20; age range: 60–79 years, median age: 71 years) were included in this study. Serum T and PSA concentrations before surgery were available for all patients. None of the patients had been treated with a 5ARI before surgery. Histopathological samples were reviewed by two experienced pathologists (HXB and QZ) to ensure that the histological entities were clearly identified and not contaminated by prostate cancer. This study was approved by the ethics committee of the Jing'an District Central Hospital (approval No. 2020-05). Informed consent was obtained from all patients.

Materials

DHT was purchased from APEXBIO (Houston, TX, USA). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TNF-α inhibitor R7050 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Enzyme-linked immunosorbent assay (ELISA) kits and antibodies against cyclin D1, B-cell lymphoma-extra large (Bcl-xL), p65, phosphorylated p65 (p-p65), and caspase 9 were purchased from Proteintech (Rosemont, IL, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase, B-cell lymphoma-2-associated X (Bax), **c**ellular Myc (c-Myc), and phosphorylated c-Myc (p-c-Myc) were purchased from Abmart (Shanghai, China). Antibodies against AR, p38, and phosphorylated p38 (p-p38) were purchased from Affinity Biosciences (Cincinnati, OH, USA). Mouse anti-human CD163, mouse anti-human CD11b, and mouse anti-human CD86 antibodies were purchased from Invitrogen (Shanghai, China).

Preparation of drugs and cell culture

DHT was dissolved in dimethyl sulfoxide, which was stored at −20°C. The final culture medium had a dimethyl sulfoxide concentration of ≤0.1%. LPS was dissolved in phosphate-buffered saline to prepare 100 μg ml^{-1} stock solution. The human monocytic leukemia cell line THP-1 and the immortalized prostate stromal fibroblast cell line WPMY-1 were kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The immortalized prostate epithelial cell line BPH-1 and the immortalized prostate epithelial cell line RWPE-1 were kindly provided by the Laboratory of Urology Center, Shanghai General Hospital (Shanghai, China). THP-1, WPMY-1, RWPE-1, and BPH-1 cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

Preparation of conditioned media

THP-1 cells were seeded in THP-1 medium on six-well plates (5 × 10^5 cells per well) and treated with 20 ng ml^{-1} phorbol-12-myristate-13-acetate for 48 h to induce differentiation into macrophages as described previously.[14,15] The macrophages were washed three times before adding LPS (20 ng ml^{-1}) and interferon-γ (20 ng ml^{-1}) to induce the M1 phenotype. After 24 h of culture, the cells were treated with 20 ng ml^{-1} LPS, 10 nmol l^{-1} DHT or a combination of LPS and DHT. The supernatant was collected and filtered using 0.2-mm filters and then stored at −80°C. The supernatant of THP-1 cells was designated as conditioned medium (CM), conditioned medium with LPS (CM-LPS), conditioned medium with DHT (CM-DHT), and conditioned medium with LPS+DHT (CM-LD).

Cell viability and proliferation assay

The cells were seeded into 96-well plates at a density of 2 × 10^3 cells per well and cultured for 24 h or 72 h. At the end of each time point, 10 μl of Cell Counting Kit-8 (CCK-8) reagent was added to each well, and the cells were incubated for 3 h. The optical density of each well was measured at a wavelength of 450 nm. The CCK-8 assay in each experiment was carried out in triplicate.

Flow cytometry

Surface marker expression in M1-polarized THP-1 cells was analyzed by flow cytometry. After 72 h of polarization, the cells were washed three times with phosphate-buffered saline, gently scraped, and transferred into FACS tubes. The cells were then stained with fluorochrome-tagged monoclonal antibodies against surface CD11b, CD86, and CD163 to identify the M1 phenotype as described previously.[11] The cells were washed, suspended in cell staining buffer, and analyzed with a FACSCanto II flow cytometer (ThermoFisher, Waltham, MA, USA). The data were analyzed using FlowJo software (Becton Dickinson, Franklin Lakes, NJ, USA). Isotype-matched controls were used as a baseline reference. Typically, 2% positive cells were allowed beyond the statistical marker in appropriate controls.

ELISA assay

The production of TNF-α by M1 macrophages treated with or without LPS and DHT was measured by ELISA. THP-1 cells were seeded on six-well plates (5 × 10^5 cells per well) and treated with 20 ng ml^{-1} phorbol-12-myristate-13-acetate for 48 h. The macrophages were washed three times before adding interferon-γ (20 ng ml^{-1}) and LPS (20 ng ml^{-1}) to induce the M1 phenotype. After 24 h of culture, the cells were treated with 20 ng ml^{-1} LPS, 10 nmol l^{-1} DHT, or a combination of LPS and DHT and cultured for another 2 h, 3 h, 4 h, or 6 h. The supernatant was collected at the end of each time point and stored at −80°C until further use. Specific ELISA kits were used to measure TNF-α concentrations in the supernatant by following the manufacturer's instructions.

Western blot analysis

M1 macrophages were treated with or without LPS and DHT for 2h on six-well plates to evaluate the total protein content. WPMY-1 cells were seeded in Dulbecco's modified eagle's medium (DMEM) with 8% fetal bovine serum on six-well plates (2 × 10^5 cells per well). After

24 h of incubation for stability, the medium was changed to serum-free DMEM for starvation. After another overnight incubation, the medium was changed to DMEM with fetal bovine serum (8%), CM (2%), CM-LPS (2%), CM-DHT (2%), or CM-LD (2%). The cells were lysed, and the protein concentration was quantified by the bicinchoninic acid protein assay kit (Epizyme, Shanghai, China) in accordance with the manufacturer's instructions. Western blot analysis was performed as previously described.[16]

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA from WPMY-1 cells was extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. Total RNA was subjected to reverse transcription using PrimeScript™ RT Master Mix (Takara, Kusatsu, Japan). Quantitative real-time RT-PCR was conducted using the Roche LightCycler480II real-time PCR System (Roche, Basel, Switzerland) to determine mRNA expression levels. cDNA was amplified by PCR with specific primers (**Supplementary Table 1**). Expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA, and the data were analyzed by using the $2^{-\Delta\Delta Ct}$ method.[17]

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4 μm) of the prostate tissue from 20 patients were immunostained for TNF-α. For a negative control, the primary antibody was omitted. The mean optical density (MOD) of each section was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). Preference settings were performed as described previously.[18] Batch processing was applied by macroprocessor Pathology 6 (Media Cybemetics). The area, mean density, and integrated optical density were measured.

Statistical analyses

ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the intensity of western blot bands. Statistical analyses were carried out using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). All experiments were repeated at least three times. Data are shown as the mean ± standard deviation (s.d.). Differences between groups were analyzed by the two-sided Student's *t*-test or one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Combined treatment with LPS and DHT increases inflammation and enhances creation of M1 phenotype macrophages by TNF-α

THP-1 cells were pretreated to generate M1 macrophages (**Figure 1a** and **1b**). Flow cytometry showed M1 differentiation with CD11b and CD86 expression. CD163 staining showed that the differentiated cells did not contain M2 phenotype macrophages (**Figure 1c–1e**). The differentiated M1 macrophages were treated with LPS and DHT. We found that p-p65 was significantly activated in M1 macrophages treated with LPS and DHT compared with the other groups ($P < 0.05$; **Figure 2a–2c**). AR expression in M1 macrophages did not change (data not shown). Combined treatment with LPS and DHT for 4 h significantly stimulated M1 macrophages to release TNF-α ($P < 0.05$; **Figure 2d**) compared with LPS or DHT treatment alone, but it did not affect IL-1α expression (**Figure 2e**). Using western blot, we examined the expression of the classic p65 nuclear factor-kappa B (NF-κB) inflammation pathway.
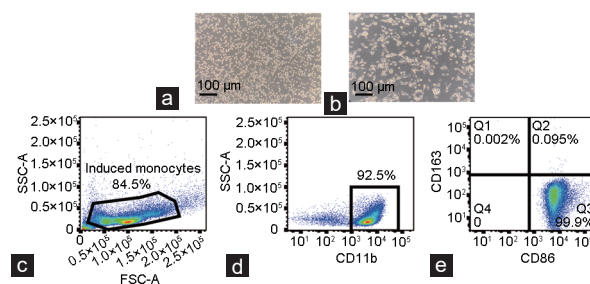

Figure 1: Differentiation of THP-1 cells to M1 macrophages. The morphological change of THP-1 cells (**a**) before and (**b**) after differentiation (40×). (**c**) The flow cytometry picture of induced THP-1 cells, (**d**) macrophage gated from induced monocytes, and (**e**) the distribution of M1 macrophage (CD86⁺CD163⁻) and M2 macrophage (CD86⁻CD163⁺) gated from macrophage cells. SSC-A: side scatter-area; FSC-A: forward scatter-area; Q1: quadrant 1; Q2: quadrant 2; Q3: quadrant 3; Q4: quadrant 4.

Conditioned medium from combined LPS- and DHT-treated M1 macrophages promotes proliferation of WPMY-1 cells

We performed the CCK-8 assay to examine the involvement of inflammatory cytokines in the proliferation of prostate cells. We assessed the proliferation of WPMY-1, RWPE-1, and BPH-1 cells, which were incubated in CM (2%), CM-LPS (2%), CM-DHT (2%), or CM-LD (2%) for 72 h. Treatment with CM-LPS, CM-DHT, and CM-LD promoted WPMY-1 prostate stromal cell proliferation (**Figure 3a**). Among them, the CM-LD treatment showed the strongest proliferative effect compared with the other two treatments. However, these effects were not observed in RWPE-1 prostate epithelial cells or BPH-1 prostate epithelial cells (**Figure 3b** and **3c**). Furthermore, the expression of proliferation- and apoptosis-related proteins, c-Myc, p-c-Myc, Bcl-xL, and Bax in WPMY-1 cells was measured by western blot (**Figure 3d** and **3e**). Protein expression levels of p-c-Myc and Bcl-xL were significantly higher in the presence of CM-LPS and CM-LD compared with those in the control group (all $P < 0.05$; **Figure 3f** and **3h**). The CM-LD treatment showed more robust proliferation-promoting effects than the CM-LPS treatment. Expressions of c-Myc and Bax, an apoptosis-related protein, were similar among the groups (**Figure 3g** and **3i**). Caspase 9 and caspase 12, classic genes involved in cell apoptosis, were not detected by western blot and real-time RT-PCR (data not shown). These results indicated that pro-inflammatory cytokines released into the CM by M1 macrophages upon LPS stimulation increased the viability of WPMY-1 prostate stromal cells, while DHT pretreatment promoted these effects.

Increased secretion of TNF-α activates the p38 and NF-κB pathways in WPMY-1 cells

The M1 macrophage pro-inflammatory response attributed to treatment with LPS and DHT increased the secretion of TNF-α (**Figure 2d**). This response likely triggered prostate stromal cell downstream signaling pathways, increasing protein expression and phosphorylation, and ultimately promoting the proliferation of prostate stromal cells. We used western blot to detect TNF-α-related protein expression levels involved in promoting proliferation and tissue regeneration pathways in prostate stromal fibroblast cells such as the p38 mitogen-activated protein kinase (MAPK) and p65 NF-κB pathways (**Figure 3j**). Both p38 and p65 protein expression levels were higher in the CM-LD group than those in the control group (both $P < 0.05$; **Figure 3l** and **3n**). Additionally, p-p38 and p-p65 were significantly upregulated in the CM-LD group (both $P < 0.05$; **Figure 3k** and **3m**), which suggested a significant activation of the MAPK and NF-κB pathways. AR expression
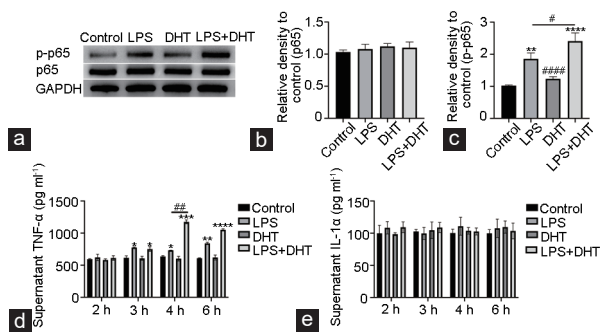
Figure 2: Combinational treatment of DHT and LPS activated p65 NF-κB pathway and increased the expression of TNF-α in M1 macrophages. M1 macrophages were treated for 24 h with 10 nmol l^{-1} DHT and 20 ng ml^{-1} LPS for 2 h. (**a**) p-p65 and p65 levels were detected by western blot. (**b**) p65 and (**c**) p-p65 protein levels in WPMY-1 cells under different culture conditions. Protein band intensity was normalized to GAPDH and is expressed as the fold difference relative to the control group. (**d**) TNF-α and (**e**) IL-1α levels in the culture supernatant were measured by enzyme-linked immunosorbent assay. The data represent the three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, LPS group, DHT group and LPS+DHT group vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#\#}P < 0.0001$, LPS group and DHT group vs LPS + DHT group. DHT: dihydrotestosterone; LPS: lipopolysaccharide; NF-κB: nuclear factor-kappa B; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TNF-α: tumor necrosis factor alpha; p-p65: phosphorylated p65; IL-1α: interleukin 1α.

in WPMY-1 cells stimulated by CM was also examined. Real-time RT-PCR and western blot analysis showed that AR expression was increased under incubation with CM-LPS, CM-DHT, and CM-LD (all $P<0.05$; **Figure 3o–3q**).

The TNF-α antagonist R7050 reverses the conditioned medium's effect on proliferation of WPMY-1 cells

To further investigate whether TNF-α-mediated NF-κB and MAPK signals are required for proliferative effects on prostate stromal cells, we assessed the effect of R7050, a selective TNF-α inhibitor, on CM-LD-treated WPMY-1 cells. First, we used the CCK-8 assay to assess R7050 cytotoxicity in WPMY-1 cells. R7050 concentrations ≤0.8 nmol l^{-1} showed no significant cytotoxicity on WPMY-1 cells, while cell numbers were significantly reduced at concentrations ≥1 nmol l^{-1} (**Figure 4a**). Therefore, all subsequent experiments were performed using 0.8 nmol l^{-1} R7050. WPMY-1 cells were seeded in six-well plates, incubated with 0.8 nmol l^{-1} R7050, and then treated with 2% CM-LD. Compared with CM-LD alone, additional treatment with R7050 significantly inhibited the proliferation of WPMY-1 cells ($P < 0.05$; **Figure 4b**) and significantly downregulated the proliferation-related proteins Bcl-xL, c-Myc, and p-c-Myc (all $P < 0.05$; **Figure 4c** and **4e–4g**). Additional treatment with R7050 also neutralized activation of the NF-κB and MAPK signaling pathways in WPMY-1 cells. Both p38 and p65 activation were significantly repressed after incubation with R7050, and p-p38 and p-p65 were downregulated compared with the CM–LD alone (all $P < 0.05$; **Figure 4d** and **4h–4k**). These results indicated that TNF-α released into the culture medium by M1 macrophages upon combined stimulation with LPS and DHT increased the viability of WPMY-1 prostate stromal cells. AR protein expression was not regulated by R7050 in prostate stromal fibroblast cells (**Figure 4l–4m**). We also performed the same treatment of R7050 in prostate epithelial cell lines. We found that R7050 barely affected the proliferation of RWPE-1 and BPH-1 cells (**Figure 4n–4o**).
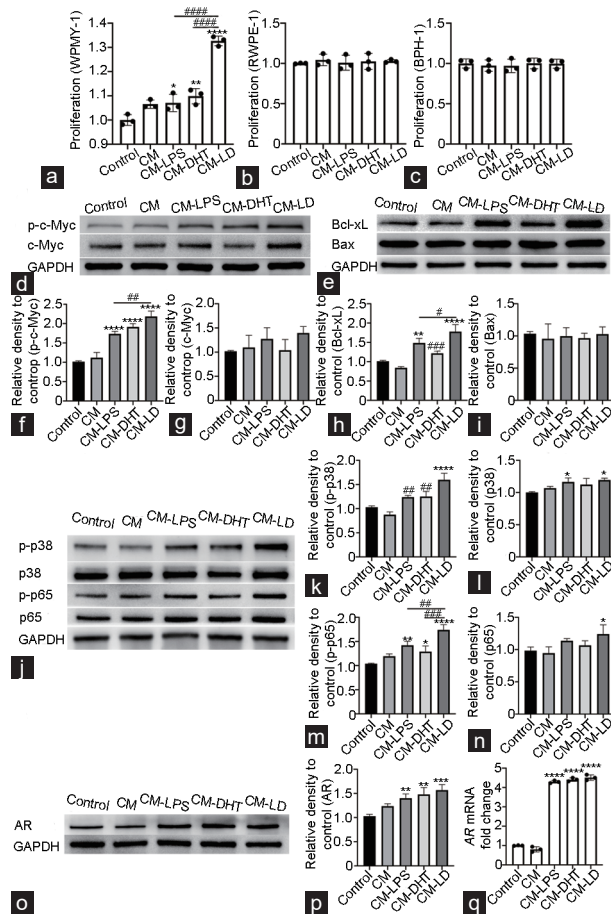

Figure 3: Conditioned media from DHT and LPS treated M1 macrophages promoted the proliferation of WPMY-1 prostate fibroblast stromal cells and activated the TNF-α regulated cell proliferation and tissue regeneration pathways. (**a**) WPMY-1, (**b**) RWPE-1 and (**c**) BPH-1 cells were incubated with 2% CM for 72 h and proliferation was measured by CCK-8 assay. WPMY-1 cells were incubated with conditioned media for 6 h, proliferation and apoptosis related protein (**d**) p-c-Myc and c-Myc, and (**e**) Bcl-xL and Bax levels were detected by western blot. (**f**) p-c-Myc, (**g**) c-Myc, (**h**) Bcl-xL and (**i**) Bax protein levels in WPMY-1 cells under different culture conditions. (**j**) TNF-α regulated cell proliferation and tissue regeneration pathways (p-p38, p38, p-p65 and p65) were detected by western blot. (**k**) p-p38, (**l**) p38, (**m**) p-p65 and (**n**) p65 protein levels in WPMY-1 cells under different culture conditions. AR expression of WPMY-1 incubated with conditioned media was detected (**o** and **p**) by western blot and (**q**) quantitative real-time reverse transcription polymerase chain reaction. The data represent the three independent experiments. Protein band intensity was normalized to GAPDH and is expressed as the fold difference relative to the control group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, CM group, CM-LPS group, CM-DHT group and CM-LD group vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$, CM group, CM-LPS group and CM-DHT group vs CM–LD group. DHT: dihydrotestosterone; LPS: lipopolysaccharide; CCK-8: cell counting Kit-8; AR: androgen receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TNF-α: tumor necrosis factor alpha; c-Myc: cellular Myc; Bcl-xL: B-cell lymphoma-extra large; Bax: B-cell lymphoma-2-associated X; p-p65: phosphorylated p65; p-p38: phosphorylated p38; p-c-Myc: phosphorylated c-Myc; CM: conditioned medium; CM-LPS: conditioned medium with LPS; CM-DHT: conditioned medium with DHT; CM–LD: conditioned medium with LPS+DHT.

TNF-α expression is higher in patients with a larger PV and is related to high serum T and PSA concentrations in patients with BPH

Twenty histopathological samples were reviewed by two experienced pathologists to ensure that hyperplasia of the prostate was clearly identified and not contaminated by prostate cancer. We used a PV of

60 ml as the cutoff value, and the 20 cases of BPH were divided into two groups as follows. Ten patients with PV >60 ml were classified as the large group, and 10 patients with PV ≤60 ml were classified as the small group (**Supplementary Table 2**). General data of patients with BPH in two groups are shown in **Table 1**. There were no significant differences in the patients' general characteristics, except for the PV, between the two groups. Immunohistochemistry showed that TNF-α was expressed in glandular epithelium and mesenchymal tissue (**Figure 5a** and **5b**). The MOD value of TNF-α expression was higher in the large group (mean ± s.d.: 0.0058 ± 0.0070) than that in the small group (mean ± s.d.: 0.0007 ± 0.0009, $P < 0.05$; **Figure 5c**).

The correlations of MOD with serum T and PSA concentrations were also analyzed in this study. Using a serum T concentration of 12 nmol l^{-1} as the cutoff value, the 20 patients were divided into the two following groups: patients with T concentrations >12 nmol l^{-1} were assigned 1 point, and those with T concentrations ≤12 nmol l^{-1} were assigned 0 point. Similarly, using a serum PSA concentration of

4 ng ml^{-1} as the cutoff value, the 20 patients were divided into those with PSA concentrations >4 ng ml^{-1} (1 point) and those with PSA concentrations ≤4 ng ml^{-1} (0 point). There was no significant difference in MOD values between different PSA scores ($P = 0.1348$; **Figure 5d**) and T scores ($P = 0.1349$; **Figure 5e**). However, after combining the T and PSA scores, MOD values of patients with BPH were significantly higher in patients with 2 points than those in patients who scored 0 and 1 point ($P = 0.0010$; **Figure 5g**). Interestingly, when we analyzed the data in the large group only, there was a significant difference in MOD values between different T scores ($P = 0.0481$; **Figure 5f**).

DISCUSSION
BPH is a condition involving hyperplasia of prostatic tissue, especially of stromal tissue in the transition zone.[5] Although the basic mechanisms of BPH remain unclear, androgens and inflammation are thought to be important factors that lead to the pathological changes in BPH. The direct stimulation effect of inflammation or DHT on prostate tissue has been reported by numerous studies.[1,10] However, few studies have examined the synergy between DHT and inflammation in jointly promoting the development and progression of BPH. In this study, for the first time, we performed a detailed investigation of the pro-inflammatory effects of DHT and LPS in M1 macrophages and studied their effects on the proliferation of human prostate cells. We also analyzed the relationships between PV, androgens, PSA concentrations, and TNF-α expression in patients with BPH.

The prostate is a sex-steroid hormone-dependent organ. In prostate tissue, DHT is a potent metabolite and is vital for gland

Table 1: General data of benign prostatic hyperplasia patients with different prostate volume (mean±standard deviation)

Characteristic	Small group	Large group	P
PV (ml)	42.73±14.22	95.57±37.80	0.0006*
Age (year)	71.5±5.5	70.6±6.1	0.6242
Serum T (nmol l^{-1})	14.22±3.55	12.52±2.73	0.1830
PSA (ng ml^{-1})	4.99±4.96	10.18±7.70	0.0630

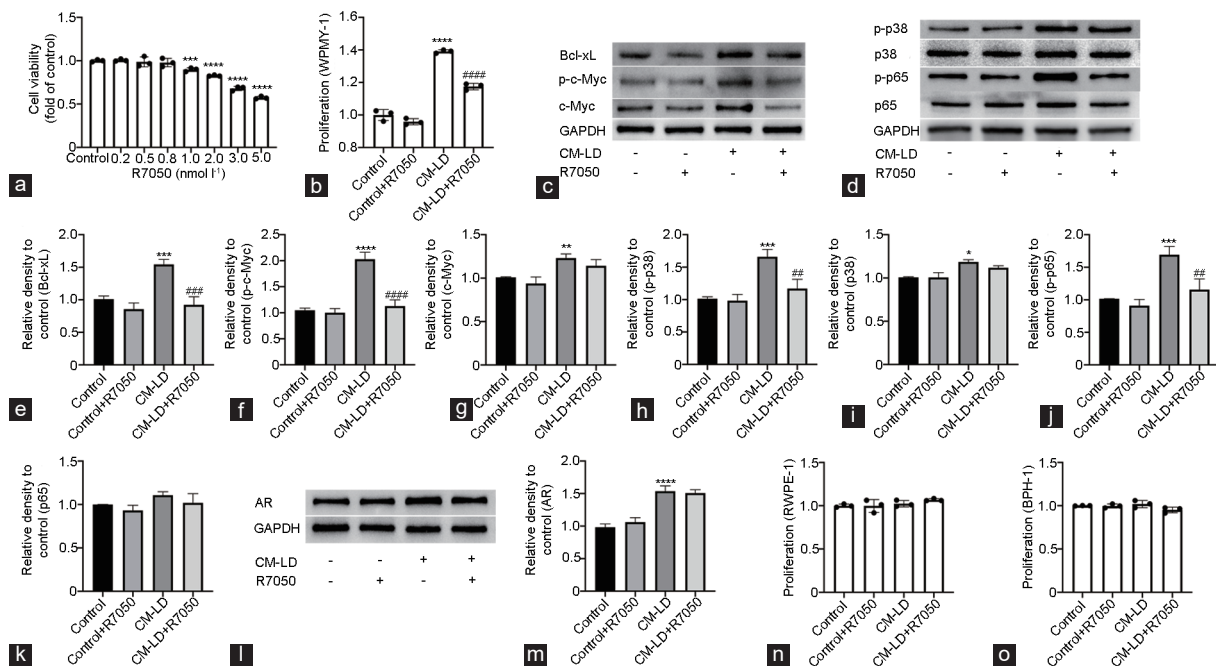*P<0.05. PV: prostate volume; T: testosterone; PSA: prostate-specific antigen

Figure 4: TNF-α antagonist R7050 reversed conditioned medium effect of proliferation in WPMY-1 cells, but had no influence on AR expression. (**a**) WPMY-1 cells were treated with various concentrations of R7050 for 24 h to test the effect of R7050 on the viability of WPMY-1 cells. (**b**) WPMY-1 cells were treated with 2% CM-LD and 0.8 nmol^{-1} R7050 for 72 h. Both cell viability and proliferation were measured by CCK-8 assay. The effect of R7050 on the expression of CM-LD related protein (**c**) Bcl-xL, p-c-Myc and c-Myc, and (**d**) p-p38, p38, p-p65 and p65 levels were detected by western blot. (**e**) Bcl-xL, (**f**) p-c-Myc, (**g**) c-Myc, (**h**) p-p38, (**i**) p38, (**j**) p-p65 and (**k**) p65 protein levels in WPMY-1 cells under different culture conditions. (**l**) AR expression of WPMY-1 incubated with conditioned media was detected by western blot. (**m**) AR protein levels in WPMY-1 cells under different culture conditions. (**n**) RWPE-1 cells and (**o**) BPH-1 cells were treated with 2% CM-LD and 0.8 nmol l^{-1} R7050 for 72 h. Protein band intensity was normalized to GAPDH and is expressed as the fold difference relative to the control group. *$P < 0.05$, ***$P < 0.001$ and ****$P < 0.0001$, CM-LD group *vs* control group; ##$P < 0.01$, ###$P < 0.001$, and ####$P < 0.0001$, CM-LD+R7050 group *vs* CM-LD group. DHT: dihydrotestosterone; LPS: lipopolysaccharide; CCK-8: cell counting Kit-8; AR: androgen receptor; GAPDH: glyceraldehyde phosphate dehydrogenase; c-Myc: cellular Myc; Bcl-xL: B-cell lymphoma-extra large; p-p65: phosphorylated p65; p-p38: phosphorylated p38; p-c-Myc: phosphorylated c-Myc; CM-LD: conditioned medium with LPS+DHT.
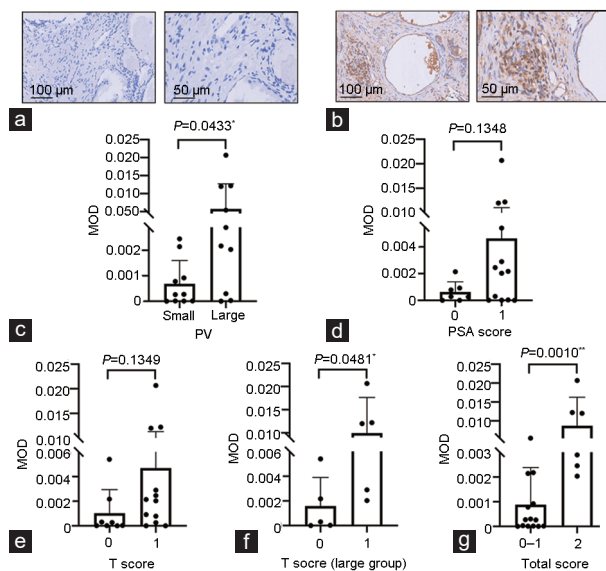
Figure 5: The relationship between TNF-α expression and prostate volume, serum T level and PSA level in the BPH specimen. (**a**) Without and (**b**) with immunohistochemistry staining of TNF-α. (**c**) Large PV is associated with TNF-α expression. (**d**) PSA level and (**e**) T level showed no association with TNF-α expression. (**f**) Among the patients in the large group (PV >60 ml), higher T level is associated with TNF-α expression. (**g**) By adding T score and PSA score, patients with higher T level and PSA level are more likely have higher TNF-α expression. *P* values generated from Mann–Whitney U test or 2-tailed Student's *t*-test. *$P < 0.05$, **$P < 0.01$. TNF-α: tumor necrosis factor alpha; PV: prostate volume; T: testosterone; PSA: prostate-specific antigen; BPH: benign prostatic hyperplasia; MOD: mean optical density.

development, function, and progression.[4] Inhibiting the synthesis of DHT is an effective treatment for BPH.[19] BPH is also thought to be an inflammation-related disease.[1] Macrophages, which are found in 82% of prostatectomy samples, are generally divided into two types as follows. M1 macrophages, which are pro-inflammatory, are characterized by high expression levels of pro-inflammation factors such as TNF-α, interleukin (IL)-1, IL-12, chemokine (C-X-C motif) ligand 10, and inducible nitric oxide synthase. M2 macrophages, which are anti-inflammation and immunoregulatory, are characterized by high expression levels of transforming growth factor-β and IL-10.[11,20] M1 macrophages have strong antimicrobial activity and mediate tissue damage and also diminish tissue regeneration and wound healing, which are highly consistent with the pathological changes of BPH. Investigation of the relationship between DHT and inflammation in BPH will help to understand the pathogenesis of prostate diseases and enable the creation of better treatment plans in the clinical setting.

Previous studies focusing on the relationship between androgens and inflammation showed confusing results. Quintar *et al.*[21] studied rats with androgen deprivation (orchiectomy) and found that the expression of intrinsic immune-related proteins and antibacterial proteins in prostate tissue was increased. By analyzing the results of the PCPT, Murtola *et al.*[12] found that the incidence of inflammation in the prostate was higher after taking finasteride in the benign and malignant groups compared with the control group, which suggested that DHT inhibited the occurrence of inflammation.[12] In contrast, the classic NF-κB inflammation pathway was activated in prostate tissue when constructing a BPH mouse model using exogenous androgens, and TNF-α, IL-8, and cyclooxygenase-2 expression were greatly upregulated.[22,23] Patients with hypogonadism, even accompanied by

prostate inflammation and metabolic syndrome, have a smaller PV compared with healthy people.[24,25] T replacement therapy was found to significantly increase the PV in hypogonadal patients with a waist circumference ≥94 cm.[24] The relationship between androgens and inflammation is still unclear. These previous studies suggest that general inflammation alone is not sufficient to increase the PV or promote the progression of hyperplasia of the prostate. In this context, the combination of DHT and inflammation might be more effective in accelerating prostate growth.

Zhao *et al.*[26] found that DHT increased TNF-α expression in human macrophages activated by LPS. These pro-inflammatory effects were mediated by AR. Similar results were observed in our study, in which DHT promoted inflammation in macrophages, activated the NF-κB pathway, and led to the increased secretion of TNF-α. TNF-α is a potent inflammatory mediator exerting various effects in the pathogenesis of various chronic inflammatory diseases including rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, psoriatic arthritis, and inflammatory bowel disease.[27] Xia *et al.*[28] found that serum TNF-α concentrations were significantly increased in patients with BPH, and were positively correlated with the PV and prostate symptom scores. Additionally, during 4 years of follow-up, the incidence of new BPH in patients with elevated TNF-α concentrations was significantly higher than that in the control group. These previous findings suggested that TNF-α may serve as a more reliable marker of BPH than other inflammation factors. We examined TNF-α expression in BPH in our study. We found that TNF-α protein expression was significantly higher in the prostate with a PV >60 ml, which suggested a potential relationship between prostatic TNF-α expression and hypertrophy of the prostate. We also examined the relationship of TNF-α protein expression and serum T and PSA concentrations. Because DHT concentrations in the prostate are not linearly related to serum T concentrations, saturation occurs during the conversion of T to DHT. We chose the lower limit of the normal value of serum T (12 nmol l^{-1}) as the cutoff to analyze the relationship between androgens and TNF-α. Similarly, the serum PSA concentration is also considered as a good indicator of the PV.[5] In 1524 men, Bohnen *et al.*[29] observed that PSA was useful for detecting an enlarged benign prostate (PV >30 cm³; with more accuracy for a PV >40 cm³ and >50 cm³). PSA concentrations (>4.0 ng ml^{-1}) are better for discriminating a large PV and can be an efficient way to distinguish patients with an enlarged prostate for men without prostate cancer.[29] This study showed that patients with BPH whose serum T concentrations exceeded 12 nmol l^{-1} combined with PSA concentrations >4 ng ml^{-1} had significantly higher TNF-α expression compared with patients who did not show these high concentrations. Additionally, the T or PSA concentration alone was not significantly related to TNF-α expression. These results indicated that an androgen alone is not sufficient to cause upregulation of TNF-α expression. More precision in estimating TNF-α expression and the PV might be obtained when using a formula containing serum T and PSA concentrations. Prospective studies are required to evaluate the effects of TNF-α on hypertrophy of the prostate and the accuracy of T and PSA concentrations for predicting the pathological results of TNF-α expression.

TNF-α binds to membrane TNF receptors, inducing activation of several signaling pathways and a broad-spectrum signaling cascade. The NF-κB and p38 MAPK pathways are the best-characterized components that promote cell proliferation and tissue regeneration.[30] Activating NF-κB signaling leads to the transcription of anti-apoptotic factors, such as Bcl-xL. R7050, which is a small-molecule TNF-α inhibitor that selectively blocks TNF-α

function by inhibiting intracellular events, is a robust tool used for examining anti-TNF-α therapy.[31] In this study, DHT- and LPS-treated M1 macrophages secreted elevated TNF-α expression. The supernatant of co-treated macrophages promoted the proliferation of human prostate stromal fibroblast cells and activated the NF-κB and p38 MAPK pathways, while R7050 effectively neutralized the proliferative effects of CM-LD. In contrast, the supernatant had no effect on prostate epithelial cells, which suggested a pivotal role of TNF-α in progressive proliferation of stromal tissue. Middleton *et al*.[32] analyzed the difference in gene expression in the normal prostate, the BPH prostate, and BPH stromal nodules. They found that CD163+ cells (macrophages) were significantly increased in BPH and BPH stromal nodules, and macrophage gene was prominent in BPH stromal nodules. Nonetheless, macrophage features were not significantly higher in the BPH prostrate compared with the normal prostate.[32] These findings indicate that macrophages have a more pronounced effect on BPH stromal nodules than on the rest of the tissue.

Some findings in our study require further investigation. We observed increased AR expression in prostate stromal fibroblast cells after CM-LPS, CM-DHT, and CM-LD treatment, and these effects were not neutralized by R7050. These results indicated that TNF-α does not regulate AR expression, and the AR pathway does not participate in the regulation of cell proliferation caused by different conditioned media. Although the causes of upregulation of AR expression remain unclear, previous studies[5,33] have shown that AR plays a pivotal role in prostate diseases. Therefore, increased expression of AR may further enhance the proliferative effects of DHT in the prostate.

CONCLUSIONS
This study shows that DHT promotes the proliferation of prostate stromal cells by increasing TNF-α expression in LPS-treated M1 macrophages. TNF-α protein expression is higher in patients with a large PV or patients with high serum T and PSA concentrations. Potential therapeutic strategies for specific patients with BPH may involve TNF-α.

AUTHOR CONTRIBUTIONS
YT conceived the study, participated in its design and coordination, performed the statistical analysis, and draft the manuscript. YJG contributed to data collection and helped manuscript preparation. QZ and HXB helped to perform the experiments. KK helped to perform data analysis and provided constructive discussion. RYZ helped to conceive the project and participated in coordination and draft preparation. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

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Supplementary Table 1: Primers used for quantitative real-time polymerase chain reaction in this study

Gene	PCR-F	PCR-R
AR	5'-CCAGGGACCATGTTTTGCC-3'	5'- CGAAGACGACAAGATGGACAA-3'
Caspase 9	5'-CCCTCACGCATCACACCTTAC-3'	5'-GCACACCCACTTTCCGTTTG-3'
Caspase 12	5'-AACAACCGTAACTGCCAGAGT-3'	5'-CTGCACCGGCTTTTCCACT-3'
GAPDH	5'-GTCAGTGGTGGACCTGACCT-3'	5'-ACCTGGTGCTCAGTGTAGCC-3'

GAPDH: glyceraldehyde phosphate dehydrogenase; PCR: polymerase chain reaction; AR: androgen receptor

Supplementary Table 2: Benign prostatic hyperplasia cases profiled

Case	Age	Prostate volume	Group	Serum testosterone (nmol/L)	T score	PSA (ng/mL)	PSA score	Total score	MOD
1	75	19.2	Small	16.57	1	0.88	0	1	0.00000402
2	60	25.8	Small	16.72	1	0.98	0	1	0.0009187
3	74	28.6	Small	11.76	0	2.37	0	0	0.00027331
4	76	39.9	Small	15.18	1	2.6	0	1	0.00214534
5	68	41.0	Small	15.54	1	1.77	0	1	0.00026044
6	66	48.2	Small	9.62	0	14.93	1	1	0.00001384
7	79	52.7	Small	19.17	1	2.5	0	1	0.00000628
8	72	55.9	Small	7.96	0	8.98	1	1	0.00001293
9	71	58.0	Small	16.74	1	11.49	1	2	0.00245264
10	74	58.0	Small	12.91	1	3.38	0	1	0.00078114
11	73	60.4	Large	13.96	1	7.27	1	2	0.01198437
12	65	61.3	Large	10.39	0	8.49	1	1	0.00217366
13	63	66.8	Large	8.43	0	4	1	1	0.0000309
14	60	71.7	Large	14.76	1	11.38	1	2	0.00203229
15	71	88.2	Large	9	0	5.89	1	1	0.0002969
16	67	90.9	Large	13.94	1	31.3	1	2	0.00289538
17	73	100.5	Large	16.22	1	8.84	1	2	0.01220555
18	77	103.9	Large	11.69	0	6.59	1	1	0.00000143
19	77	127.9	Large	15.46	1	8.25	1	2	0.02067298
20	76	184.1	Large	11.32	0	9.74	1	1	0.00540409

PSA: prostate-specific antigen; MOD: mean optical density