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ORIGINAL ARTICLE

Androgen depletion augments antibacterial prostate host defences in rats

A. A. Quintar,*[1] C. Leimgruber,*[1] O. A. Pessah,† A. Doll‡ and C. A. Maldonado*

*Centro de Microscopía Electrónica, INICSA-CONICET, †Cátedra de Bacteriología y Virología Médicas, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, X5000HRA, Córdoba, Argentina, and ‡Biomedical Research Unit, Research Institute Vall d'Hebron University Hospital, Barcelona, Spain

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Correspondence:
Amado A. Quintar, Centro de Microscopía Electrónica, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Pab. Biología Celular 1° piso, Enrique Barros esq. Enf. Gordillo, Ciudad Universitaria, X5000HRA Córdoba, Argentina.
E-mail: aquintar@cmefcm.uncor.edu

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[1]These authors have contributed equally to this work.

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Summary

The defence of the male reproductive tract against microorganisms is critical for fertilization. The prostate gland has been reported to express several molecules of the innate immune system. However, little information is available about how androgens may modulate host defences within the prostate. We therefore aimed to examine in the rat the expression of the TLR4 system, which is strongly involved in pathogen recognition, and the secretion of the antibacterial substances rBD-1 and SP-D after androgen withdrawal. Immunoblotting and immunocytochemical analysis revealed a time-dependent increase in these molecules after orchiectomy, with epithelial and stromal cells being an important source of prostatic host defence proteins. In view of this, we evaluated the potential improvement in antibacterial ability of the prostatic fluid from orchiectomized animals ex vivo. Only samples from rats at 5 days post-orchiectomy showed a slight inhibition of *Escherichia coli* growth. Finally, *E. coli* was inoculated into the ventral prostate of orchiectomized or control rats, with bacterial growth being counted at 5 days after infection. Animals with androgen depletion presented a lower bacterial count, and showed few histological signs of prostatic inflammation compared with controls. In vitro studies confirmed that isolated lipopolysaccharide (LPS)-treated prostatic cells in the absence of testosterone increased SP-D. Moreover, media from these cells showed a higher antimicrobial activity than supernatants from testosterone- and LPS-treated cells. Our findings indicate that testosterone maintains a reduced expression of key elements for innate immunity and diminishes the antibacterial ability of the rat prostate. These data may represent an important mechanism underlying the immunosuppressive activity of testosterone in the gland. However, this immunosuppressive function of androgens is understandable as a means of avoiding uncontrolled immune responses against the haploid male gamete in the reproductive tract.

Introduction

The integrity of the male tract against pathogenic microorganisms is critical for fertilization and the continuance of species. Therefore, antibacterial strategies and innate immunity are essential mechanisms in epithelia lining the reproductive tracts (Hall *et al.*, 2002; Collin *et al.*, 2008). Epithelial roles in innate immunity have been known since Sir Alexander Fleming reported in 1922 that lysozyme and other mucosal substances prevent the growth of bacteria (Fleming, 1922). However, only in the last decade have the molecular mechanisms of host defence at mucosal surfaces begun to be dissected, especially in epithelial cells from the airways and the digestive system. As the frontline of defence, the innate immune system has evolved several proteins as Toll-like receptors (TLRs) to sense infections (Takeda *et al.*, 2003; Kawai & Akira, 2007, 2010). These receptors recognize molecular patterns of pathogens and activate multiple signalling pathways which finally lead to nuclear translocation of NF-κB and

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the subsequent activation of antimicrobial and pro-inflammatory genes (Takeda *et al.*, 2003; Kawai & Akira, 2007).

Typically, the innate immune response to Gram-negative bacteria involves recognition of the lipopolysaccharide (LPS) by TLR4, while CD14 and MD-2 serve as the ligand-binding part of the LPS receptor complex (Kawai & Akira, 2010). Triggering of TLR4 results in the activation of the common intracellular TLR adaptor MyD88 or in an alternative pathway that relies on the Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) (Takeda *et al.*, 2003; Kawai & Akira, 2007, 2010). With the male urogenital tract representing an entry point for microorganisms from the environment, it is not surprising that TLRs, as well as other essential signalling components for host defence (CD14 and MyD88), have been found to be expressed in the testis, epididymis, vas deferens and accessory sex glands of different species (Hall *et al.*, 2002; Girling & Hedger, 2007; Palladino *et al.*, 2007; Bhushan *et al.*, 2009; Pudney & Anderson, 2010). In both humans and rats, we previously reported the presence and functionality of the TLR4 system in the prostate gland, being up-regulated in experimental bacterial prostatitis (Quintar *et al.*, 2006), as well as after LPS challenge in vitro (Gatti *et al.*, 2006, 2009; Leimgruber *et al.*, 2011). In addition, prostatic epithelial cells express other TLRs and react to bacterial compounds by activating several genes of cytokines and chemokines involved in inflammatory responses as well as antimicrobial proteins (Gatti *et al.*, 2006; Kundu *et al.*, 2008; Pei *et al.*, 2008; Paone *et al.*, 2010).

The secretion of microbicidal agents is the main effector mechanism of the host defence against pathogens. β-defensins are small (~3.5 kDa) cationic peptides, forming a family of four members in the human (HBD-1 to 4), with a broad spectrum of antimicrobial activities, and whose expression is controlled primarily by microbial products (through TLRs) and by inflammatory cytokines (Zhao *et al.*, 1996; Palladino *et al.*, 2003; Takeda *et al.*, 2003; Avellar *et al.*, 2004; Bullard *et al.*, 2008). Interestingly, epithelial cells from the male tract are an important source of β-defensins (Palladino *et al.*, 2003; Avellar *et al.*, 2004; Bullard *et al.*, 2008; Yudin *et al.*, 2008) but their expression in the prostate remains poorly understood. On the other hand, the classical antimicrobial activity of semen has been largely attributed to compounds of prostatic origin such as zinc and lactoferrin (Mardh & Colleen, 1975; Wichmann *et al.*, 1989). Moreover, we have recently reported that the antimicrobial Surfactant Protein D (SP-D) is present in the prostate gland (Oberley *et al.*, 2007), where this collectin protects prostate epithelial cells from infection by Chlamydia in vitro (Oberley *et al.*, 2005). Accordingly, the prostate gland might have a powerful innate immune system that defends male reproductive organs from microbial attack. However, little information is available about the regulation of host defence proteins and the behaviour of these molecules against pathogens within the prostate gland.

Studies dealing with androgen regulation of immune responses have mainly focused on adaptive immunity and on the sexual dimorphism existing in the incidence and/or severity of many diseases (Moller *et al.*, 1998; Martin, 2000; Spinedi *et al.*, 2002; Cutolo *et al.*, 2004; Nunn *et al.*, 2009). Several lines of evidence have established that females are more susceptible to autoimmune disorders (Jacobson *et al.*, 1997), with this occurrence being attributed to the lack of host androgen, because exogenous testosterone can reverse this tendency to autoimmune disorders in female mice (Roubinian *et al.*, 1979). Moreover, androgen deprivation augments the levels of T cells and their proliferation in response to TCR-mediated co-stimulation as well as to Ag-specific activation (Roden *et al.*, 2004). Such findings clearly demonstrate that testosterone generally blunts immunity when present. Currently, this concept is widely accepted, with the immunosuppressive effects of androgens on adaptive immunity being confirmed by different approaches. However, understanding the influence of male hormones on innate immune responses is far from solved.

Unlike many organs in the body, the prostate is under strict control by testicular male hormones. Orchiectomy causes a rapid involution of the prostate attributable to epithelial apoptosis leading to a complete cessation of the secretory functions. Strikingly, smooth muscle cells and fibroblasts of the prostatic stromal compartment change their phenotypes after androgen deprivation, augmenting their cellular activity (Antonioli *et al.*, 2004). In this context, it is likely that testosterone may influence the expression of host defences as well as the outcome of infectious and inflammatory diseases of the prostate. In a previous work, we have reported an increase of TLR4 expression in epithelial and stromal cells of the ventral prostate after castration in rats (Quintar *et al.*, 2006), which is in agreement with the putative role of testosterone on immune components. However, the effects of sex hormones on immunity are complex depending particularly on the experimental conditions they were proved (Munoz-Cruz *et al.*, 2012). Our previous data suggest that testosterone could influence the prostate innate immunity by modulating the expression of host proteins. Therefore, the present study characterized the expression of members of the TLR4 system as well as antimicrobial substances in the prostate of castrated animals. Furthermore, the potential of androgen depletion to modulate the antibacterial activity of the rat prostate was tested in vivo and in vitro.

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Materials and methods

Animals

Adult 16-week-old male rats, Wistar strain, weighing 250–300 g, were housed at the Animal Research Facility of the National University of Cordoba, in air-conditioned quarters, under a controlled photoperiod (14-h light/10-h darkness) with free access to commercial rodent food and tap water. All animal experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals and approved by a local ethical committee.

Orchiectomy and testosterone replacement

Rats were orchiectomized via the scrotal route under ketamine (80 mg/kg)/xylazine (8 mg/kg) anaesthesia; the epididymis and epididymal fat were also removed and the day of surgery was termed 0. The orchiectomized animals were divided into groups containing four rats each. One group of rats was killed at each of the following days: d 1 (C1), d 3 (C3), d 5 (C5), d 7 (C7), or d 12 (C12) after surgery. Two additional groups received Testosterone (2 mg/kg body weight/day; Sustanon, Organon) administered s.c. daily beginning at 3 days post-castration for a period of 4 days (C7T) or at 7 days after surgery for 5 days (C12T) and the animals were killed the next day. As controls, age-matched animals were sham orchiectomized and killed 7 days after surgery (S group). Three such independent experiments were performed. To confirm androgen depletion, serum total testosterone levels of individual rats were determined by electrochemiluminescence (ECL) immunoassay using Roche Elecsys E170 immunoassay analysers (Roche Diagnostics GmbH, Mannheim, Germany).

Prostate bacterial infection

To examine bacterial growth in the prostate, animals treated as those from groups C3, C5, C7, C7T and S (six rats/group) were inoculated with bacteria into the ventral prostate as previously described (Quintar *et al.*, 2006, 2010). Briefly, a strain of Uropathogenic *Escherichia coli* from patients with a complicated urinary tract infection was first grown overnight in tryptic soy broth at 37 °C, then washed and resuspended in sterile PBS at a concentration of 10^6 CFU/mL.

The rats were anaesthetized and subjected to a laparotomy to expose the ventral prostate. Infection was induced by intraprostatic injection of 100 μL of *E. coli* suspension with a 30 gauge needle. Animals (namely C3 + INF, C5 + INF, C7 + INF, C7T + INF, and S + INF) were killed at 5 days after inoculation and the ventral prostate was harvested and processed for bacterial counting and biochemical studies.

Prostatic cell culture and treatments

Ventral prostate specimens were obtained from six Wistar rats per culture. The tissues were minced in small fragments and treated for 30 min with a digestion solution containing 200 U/mL collagenase type IA (Sigma Aldrich, St. Louis, MO, USA) and 0.05% deoxyribonuclease type I (Sigma) in minimal essential medium SMEM (Sigma). The digested material was passed through a tissue sieve. Dispersion was then washed three times with SMEM; after each wash, cells were collected by centrifugation for 2 min at 1000 ***g***. These dispersed cells were analysed by light microscopy resulting a 90% of cells with epithelial morphology. Isolated cells were resuspended and adjusted to 1×10^6 cell/mL to be cultured in RPMI 1640 medium (Sigma), supplemented with 10% heat-inactivated foetal calf serum, 5% horse serum (Gibco, Invitrogen, Carlsbad, CA, USA), 5 μg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL selenium and 10 ng/mL EGF. Cell suspension was plated on 24-well culture plates in a humidified incubator at 37 °C supplied with 5% CO_2. The medium was daily replaced for 3 days, being replaced then by serum-free medium without antibiotics (ATB) and supplemented or not with testosterone for 48 h. The androgen was dissolved in absolute ethanol and diluted in RPMI media to the final concentration of 10^{-7} M. Ethanol was used at a final concentration of 0.2% as vehicle control. Alternatively, prostatic cells were stimulated with 1 μg/mL LPS (Sigma) for additional 24 h in presence or absence of testosterone. All cell culture experiments were repeated at least three times.

Immunocytochemistry

Ventral prostates were excised from the animals, fixed in 4% formaldehyde, embedded in paraffin, cut into 4 μm thick sections and stained with haematoxylin/eosin or immunostained to TLR4 (goat polyclonal at 1:300, L-14 Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD14 (rabbit polyclonal at 1:100, M-305 Santa Cruz Biotechnology), MyD88 (rabbit polyclonal at 1:60, HFL-296 Santa Cruz Biotechnology), NF-κB p65 (rabbit polyclonal at 1:1000, ab7970 Abcam, Cambridge, MA, USA), rBD-1 (rabbit polyclonal at 1:50, M-69 Santa Cruz Biotechnology), SP-D (rabbit polyclonal at 1:700, AB3434 Chemicon, Temeluca, CA, USA), CD3 (mouse monoclonal at 1:50, G4.18 BD Pharmingen, Franklin Lakes, NJ, USA), $CD11^{b/c}$ (mouse monoclonal at 1:50, OX-42 BD Pharmingen), TNF-α (rabbit polyclonal at 1:40, HyCult Biotech, Plymouth Meeting, PA, USA), and *E. coli* antigens (rabbit polyclonal at 1:250, PA1-25636 Affinity BioReagents, Golden, CO, USA).

For immunocytochemistry, slides were cleared with xylene and rehydrated in a series of descending

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concentrations of ethanol solutions. Then, samples were incubated in EDTA pH 9.0 to perform antigen retrieval using microwave pre-treatment (except for detection of MyD88 and *E. coli*). To block the endogenous peroxidase activity, slides were treated with H_2O_2 in methanol for 15 min. Sections were incubated 30 min in 10% normal goat serum (Sigma) to block non-specific binding, followed by overnight incubation with primary antibodies (diluted in 1% PBS-BSA) at 4 °C in a humidified chamber. Then the sections were incubated with a specific biotinylated secondary antibody (at 1:180 Amersham Pharmacia, Buckinghamshire, UK) and ABC complex (Vector, Burlingame, CA, USA). Diaminobenzidine (DAB Sigma) was used as a chromogen substrate for 10 min, and sections were rinsed in running water. Harris haematoxylin was used as a counterstaining solution. For negative controls, antibodies were pre-absorbed with specific blocking peptides (sc-16240 P Santa Cruz Biotechnology) or replaced by rabbit or mouse normal serum.

Ultrastructural immunocytochemistry

Rats were fixed by perfusion with 4% formaldehyde, and ventral prostate blocks were next submerged in a mixture containing 1.5% (v/v) glutaraldehyde, 4% (w/v) formaldehyde in 0.1 M cacodylate buffer for 12 h. After dehydration, prostate samples were embedded in an acrylic resin (LR-White, London Resin Corporation, Berkshire, UK) and cut with a diamond knife on a JEOL JUM-7 ultramicrotome. Thin sections mounted on 250 mesh nickel grids were incubated overnight on a drop of goat anti-TLR4 (1:500) or rabbit anti-NF-κB p65 (1:1500). Immunoreactive sites were then labelled with 16 nm colloidal gold/anti-goat or anti-rabbit IgG complex (Electron Microscopy Sciences) and examined using a Zeiss LEO 906E electron microscope. For the controls, the primary antibody was replaced with goat normal serum (Sigma), purified Goat IgG (Santa Cruz Biotechnology) or PBS–BSA. Additional controls were performed by incubating grids with a

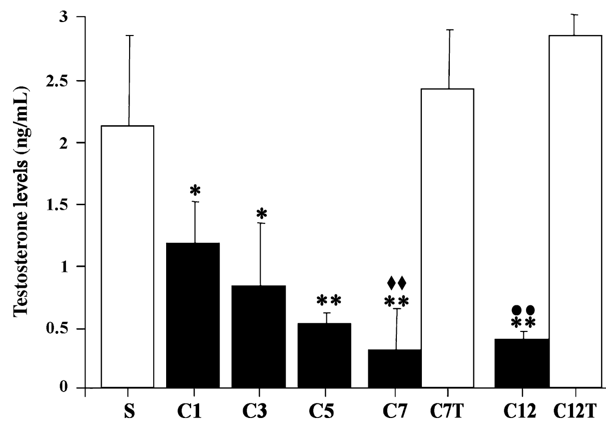
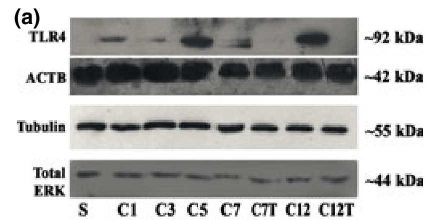
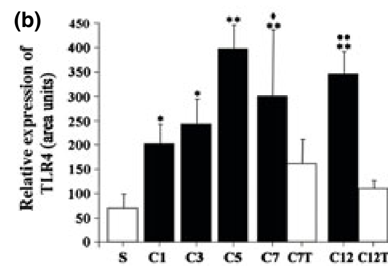


Figure 1 Effects of castration on serum levels of testosterone as determined by ECL. *vs. S $p < 0.05$, **vs. S $p < 0.01$, ♦♦ vs. C7T $p < 0.01$, •• vs. C12T $p < 0.01$ ANOVA -Tukey.





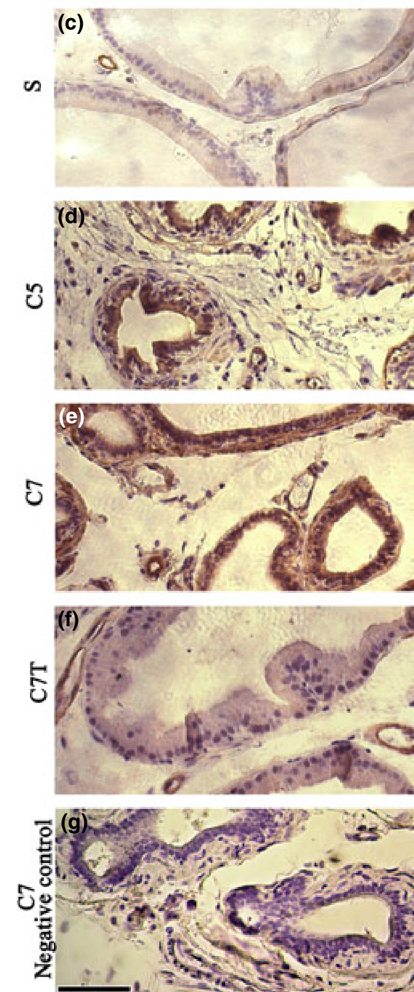


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Figure 2 Expression of TLR4 in rat ventral prostate after androgen deprivation. Representative Western blots of TLR4, along with ACTB, tubulin, and ERK (a), which were used as controls for equal protein loading in immunoblottings. Densitometric analysis of TLR4 in prostate homogenates by Western blot (b) showing a time-dependent increase after castration. Data were normalized with ACTB expression and represent the mean ± SE from at least three independent experiments. *vs. S $p < 0.05$, **vs. S $p < 0.01$, ♦ vs. C7T $p < 0.05$, •• vs. C12T $p < 0.01$ ANOVA -Tukey. Immunohistochemistry for TLR4 exhibiting an intense staining in C5 (d) and C7 (e) compared to S (c) and C7T (d) groups. As a negative control, the primary antibody was pre-absorbed with specific peptide (g). Bar = 130 μm.

solution in which the anti-TLR4 had been absorbed with its respective peptide (sc-16240 P Santa Cruz Biotechnology).

Immunoblotting

Prostate tissues or cells were minced and homogenized on ice using a teflon-glass Potter-Elvehjem tissue grinder in 2 mL cold PBS containing 1.25% Igepal CA-630, 1 mM EDTA, 2 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL aprotinin. The lysate was centrifuged at 14 000 ***g*** for 20 min at 4 °C to pellet the Igepal CA-630-insoluble material, and the supernatant was withdrawn and stored in aliquots frozen at −70 °C until required. Prostatic lysates from triplicate experimental conditions were pooled before loading into electrophoresis gels. Total protein concentration was measured with a Bio-Rad kit (Bio-Rad Protein Assay, Bio-Rad Laboratories). For Western blot, denatured protein samples (50 μg/lane) were separated on 12% SDS polyacrylamide gel and blotted to a Hybond-C membrane (Amersham Pharmacia). To assess the corresponding molecular weight, Full Range Rainbow Molecular Weight Marker was used (Amersham Pharmacia). After staining the membranes with reversible Ponceau red to check equal protein loading, incubation steps were performed in 5% defatted dry milk in PBS/0.1% Tween 20. Blots were incubated for the detection of TLR4 (goat polyclonal at 1:400, L-14 Santa Cruz Biotechnology), CD14 (rabbit polyclonal at 1:400, M-305 Santa Cruz Biotechnology), SP-D (rabbit polyclonal at 1:1000, AB3434 Chemicon) and MyD88 (polyclonal at 1:300, HFL-296 Santa Cruz Biotechnology) during 3 h. After that, membranes were treated with peroxidase-conjugated bovine antigoat or goat anti-rabbit antibodies (Jackson) and visualized applying the chemiluminescence technique. The expression of β-actin (ACTB) (mouse monoclonal at 1:5000, Sigma), α-tubulin (mouse monoclonal at 1:2000, Santa Cruz Biotechnology) and total ERK (rabbit polyclonal antibody at 1:200, Santa Cruz

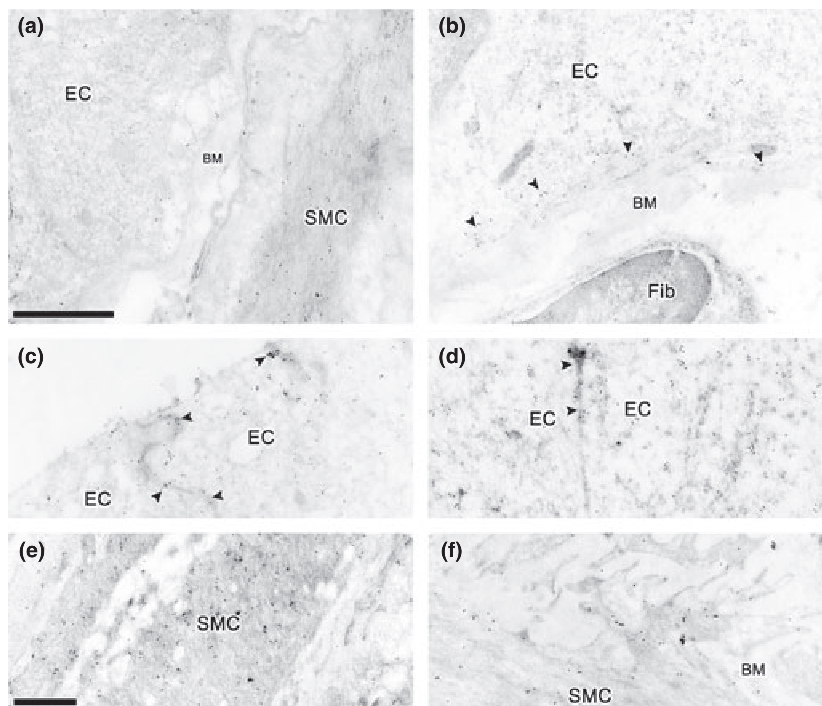

Figure 3 Ultrastructural detection of TLR4 in LRWhite-embedded prostate samples from S (a), C7 (b, c, e) and C12 (d, f) groups. Arrowheads denote gold particles labelling TLR4 next to the basal plasmalemma of epithelial cells (EC) (b) and intercellular junctions (c and d), while basement membrane (BM) appears negative. Smooth muscle cells (SMC) from castrated rats also display intense TLR4 expression in the cytoplasmic compartment (e) as well as near the cell surface of cell prolongations (f). Fib: fibroblast. Bar a–b = 2 μm, c–f = 1 μm.

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Biotechnology) were used as internal controls to confirm the equivalent total protein loading. After confirming that ACTB, ERK and α-tubulin have a statistically similar pattern expression across all samples, we used normalization with ACTB for graphics.

Rat β-defensin 1 (rBD-1) was tested in homogenates of ventral prostates by dot blot. For that purpose, prostatic lysates were matched at a concentration of 100 μg/mL, and 4 μL of each sample was spotted onto a Hybond-C Super membrane (Amersham-Pharmacia, Buckinghamshire, UK). The membrane was then treated as explained above for Western blot using an anti-HBD-1 (at 1:250, M-69 Santa Cruz Biotechnology) as primary antibody.

Semiquantitative signals were derived by densitometric analysis from Western and dot blots using Scion Image (version beta 4.0.2 Scion Corporation, Frederick, MD, USA) and data were displayed as area units.

Microbiological studies

To examine the ability of prostatic secretions from C1–C12, compared with S, C7T and C12T, to inhibit ex vivo bacterial growth, intact ventral prostate lobes from each group were first washed in ice-cold buffer, blotted onto filter paper, nicked with a sharp blade and then centrifuged at 14 000 ***g*** for 20 min to collect the prostate supernatant. This fraction (prostatic fluid) was matched at an equal concentration of total proteins among groups with a Bio-Rad kit and used to carry out the antibiogram technique as follows.

Antibiogram assay was performed by the conventional Kirby-Bauer disc diffusion test. Briefly, 6-mm filter paper discs containing 21.5 μL of prostatic fluid each were evenly spaced on Mueller Hinton agar previously flooded with Uropathogenic *E. coli* or *Staphylococcus aureus*. As positive controls for bacterial inhibition, discs embedded with streptomycin or penicillin were used. Agar plates were then incubated for 16 h at 35 °C and inhibition zones were measured from the edge of each disc. These procedures were performed in triplicate and data were analysed from at least three independent experiments.

To evaluate in vivo the growth of *E. coli* in the groups C3 + INF, C5 + INF, C7 + INF, C7T + INF, and S + INF, pieces of ventral prostate from each rat were weighed, minced and gently homogenized in tryptic soy broth (1 g of tissue in 20 mL) in sterile conditions. Then, serial dilutions, i.e. 1/5–1/50, were made and 100 μL samples of these solutions were spread on Mueller Hinton agar, with the plates being incubated overnight at 37 °C. Finally, bacterial counting was expressed as CFU per mg of prostatic tissue.

To assess in vitro the capacity of prostate cells to inhibit bacterial growth, 1 mL of supernatant was collected

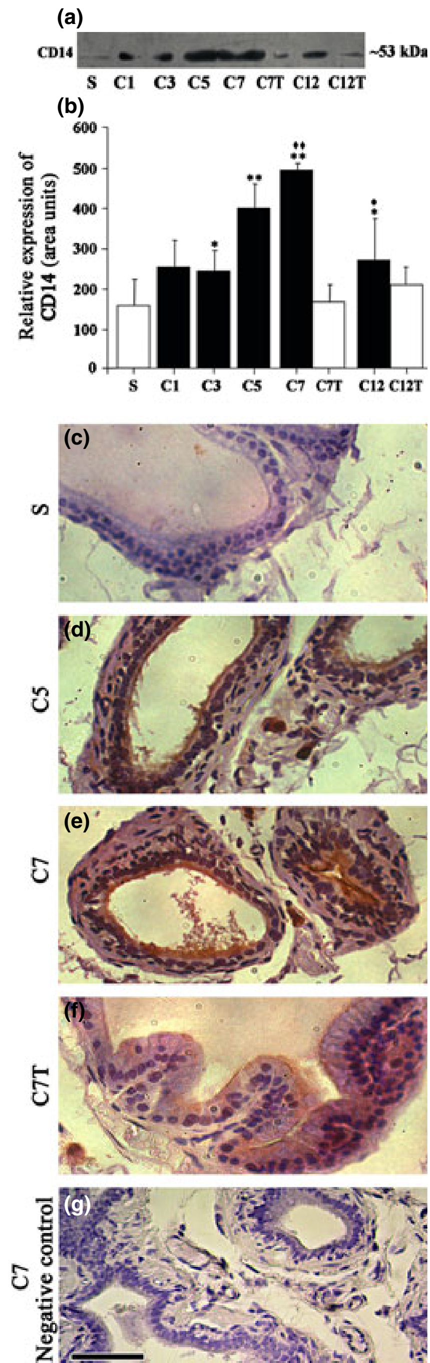

Figure 4 CD14 expression in the prostate gland after androgen suppression. A representative membrane exhibiting the CD14 signal from Western blots (a). The densitometric analysis indicates significant rises in CD14 after castration (b). Bands were normalized with ACTB and represent the mean ± SE from at least 3 independent experiments. *vs. S $p < 0.05$, **vs. S $p < 0.01$, ◆◆ vs. C7T $p < 0.01$, • vs. C12T $p < 0.05$. ANOVA -Tukey. Strong CD14 immunostaining localized at epithelial and infiltrating cells in C5 (d) and C7 (e) contrasts with the weak expression seen in S (c) and C7T (d) animals. For negative control (g), the anti-CD14 antibody was replaced by normal rabbit serum. Bar = 130 μm.

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from each plate and incubated with 1×10^4 CFU of *E. coli* for 16 h at 37 °C. Then, bacterial suspension was washed twice with sterile PBS and analysed using turbidimetric technique using a 620 nm filter.

Statistical analysis

The characterization of data was accomplished by comparing their mean values ± standard error of the mean from at least three independent protocols. Data from more than two groups were analysed using analysis of variance (ANOVA) with Tukey as the post-test to compare all pairs of columns. Statistical analyses were performed using the InStat V2.05 program from GraphPad Inc., La Jolla, CA, USA.

Results

Androgen deprivation affected the expression of the pathogen receptor TLR4 and its associated molecules CD14 and MyD88 in a time-dependent manner

To clarify the effect of testosterone on the expression of prostate host defence proteins, we used as a model orchiectomized adult rats treated (or not) with testosterone. Firstly, we validated this model by showing that androgen ablation induces a significant loss of prostate ventral weight (about 56% by 7 days after orchiectomy), while testosterone replacement allows complete restoration of prostate weight (data not shown) as well as of serum testosterone levels to sham values (Fig. 1). Prostatic acini became small, with death of epithelial cells and an important development of the periacinar smooth muscle layer. In addition, mononuclear infiltrating cells were present throughout the gland of orchiectomized animals (data not shown).

By Western blot, the expression of TLR4 increased in orchiectomized animals in a time-dependent manner, exhibiting a peak at 5 days after androgen ablation (Fig. 2a,b). In order to determine the cell expressing TLR4 in the ventral prostate, we performed an immunocytochemical analysis at both light and electron microscope levels. In control prostate, epithelial cells exhibited a weak staining (Fig. 2c) for this receptor. Interestingly, the main source of the increment of TLR4 post-orchiectomy was the stromal compartment, which becomes highly hypertrophic after deprivation of testosterone (Fig. 2d,e). Ultrastructural examination using gold particles confirmed the cells expressing TLR4 in the prostate after orchiectomy (Fig. 3).

The immune response to Gram-negative bacteria requires, apart from TLR4, co-receptors for LPS, such as CD14, and intracellular signalling molecules, such as MyD88, culminating in NF-κB translocation to the nucleus. In view of the changes in TLR4 levels in orchiectomized animals, we next aimed to analyse the expression

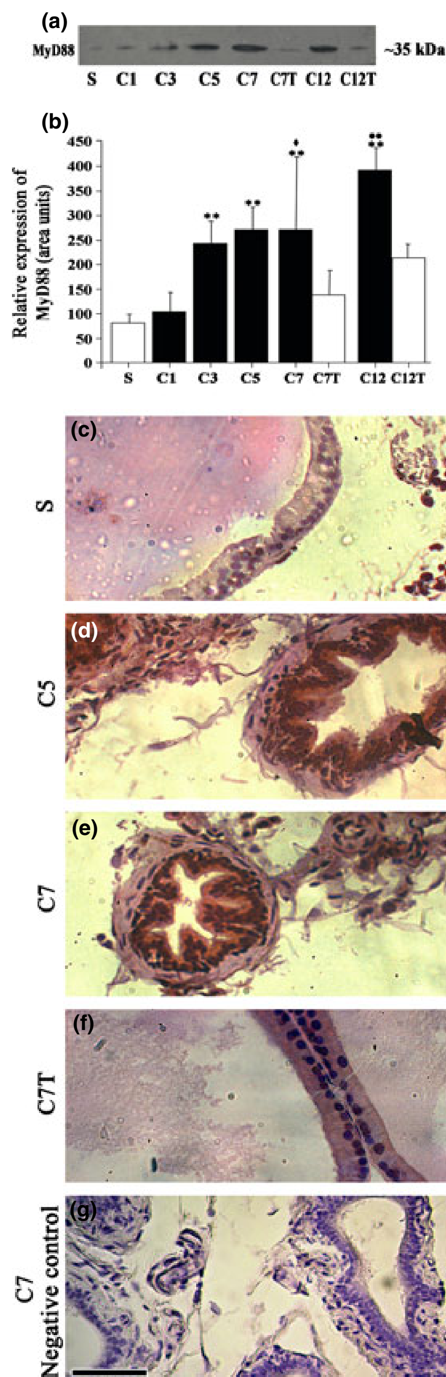

Figure 5 Prostatic levels of MyD88 after androgen depletion determined by Western blot (a) and inmunohistochemistry (b–f). Representative Western blot showing MyD88 expression (a). Densitometric analysis exhibiting a significant increase in MyD88 signal in orchiectomized rats (b); data were normalized with ACTB expression. **vs. S $p < 0.01$, ♦ vs. C7T $p < 0.05$, •• vs. C12T $p < 0.01$ ANOVA -Tukey. Intense MyD88 immunostaining is observed in epithelial cells in the prostate from C5 (d) and C7 (e), compared to S (c) and C7T (f) groups. The primary antibody was replaced by normal rabbit serum for negative controls (g). Bar = 130 μm.

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of CD14 and MyD88. The total amount of both molecules were found to be increased in the ventral prostate after androgen deprivation (Figs 4 & 5). By immunocytochemistry, the regressing epithelium displayed intense staining for CD14 by 5 days after orchiectomy (Fig. 4d), which was stronger at 7 days post-orchiectomy (Fig. 4e). Interestingly, numerous mononuclear immune cells infiltrating the gland after orchiectomy exhibited a positive reaction for this co-receptor (Fig. 4d,e). As expected, testosterone replacement restored CD14 expression to levels comparable to controls (Fig. 4a,b). In situ detection of MyD88 showed no immunoreactivity in control animals (Fig. 5c), with levels being up-regulated by 3 days post-orchiectomy and thereafter in the prostate epithelial cells (Fig. 5d,e).

Orchiectomy induced nuclear distribution of NF-κB only in stromal cells of the regressing prostate

The localization of NF-κB (subunit p65) was determined by immunocytochemistry at light and electron microscopy. Only scarce immunoreactivity was detected in the cytoplasmic compartment of prostatic epithelial cells in control animals (Fig. 6a). As shown in Fig. 6b, androgen deprivation provoked intense immunolabelling in the cytoplasm of the prostate epithelium, especially after 5 days of androgen depletion. However, NF-κB expression after orchiectomy was restricted to the cytoplasmic compartment of these cells. As a positive control, we used *E. coli*-infected prostate tissues, in which nuclear NF-κB detection was clearly detected in several epithelial cells at 48 h post-infection (Fig. 6f). Strikingly, nuclear localization of NF-κB was observed in stromal cells, particularly at 7 days post-orchiectomy (Fig. 6c,d), with this NF-κB translocation being avoided by testosterone restoration (Fig. 6e). For better cellular identification, we performed immunocytochemistry for NF-κB on LRWhite-embedded sections at ultrastructural level. This technique clearly showed that stromal cells displaying nuclear NF-κB after orchiectomy were mainly fibroblasts (Fig. 6h), which are an abundant cellular population in the prostatic stroma of these animals. Rarely, hypertrophic smooth muscle

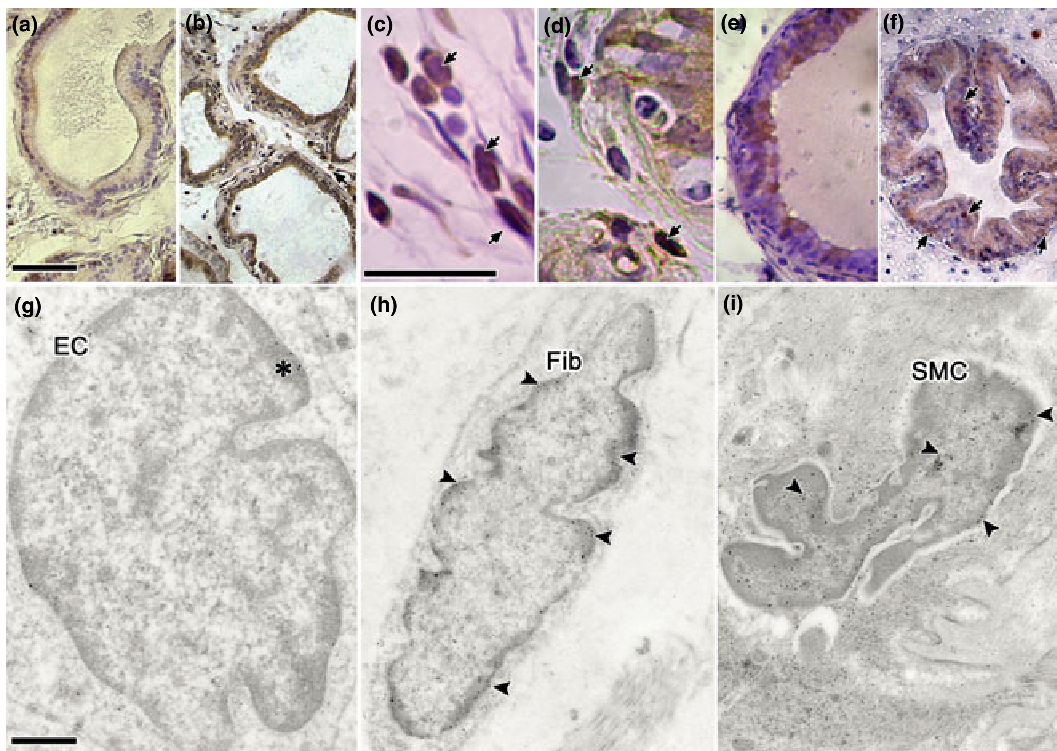

Figure 6 Immunocytochemical analysis of NF-κB at light (a–f) and electron microscopy (g–i) in prostate samples from S (a), C5 (b, c), C7 (d), and C7T (e) groups. Weak staining is observed in control rats (a), whereas orchiectomized animals clearly show nuclear localization of NF-κB in stromal cells (arrows in b, c, and d). Panel F illustrates a positive control for nuclear translocation of NF-κB in prostatic epithelial cells (arrows in f) after bacterial infection. Ultrastructural immunogold labelling evidences very scarce particles in epithelial cells (EC) (* in g), with nuclei of fibroblast (Fib) and smooth muscle cells (SMC) being strongly labelled for NF-κB in orchiectomized rats (arrowheads in h and i). Bar a, b, e, f = 100 μm, c, d = 100 μm, g–i = 1 μm.

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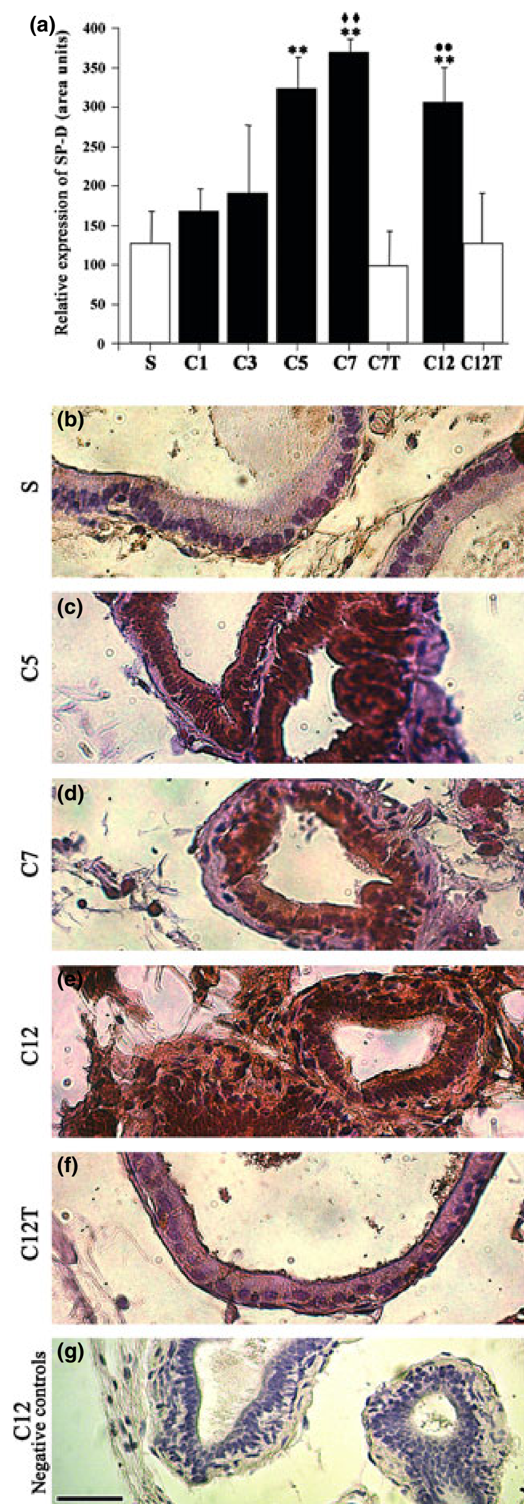

Figure 7 Expression of the antimicrobial substances SP-D in the ventral prostate. Densitometric analysis by Western blot (a) demonstrating a time-dependent increase of SP-D in orchiectomized rats. Values were normalized with ACTB expression and represent the mean ± SE from at least three independent experiments. **vs. S $p < 0.01$, ♦♦ vs. C7T $p < 0.01$, •• vs. C12T $p < 0.01$ ANOVA -Tukey. Representative immunohistochemistry pictures from S group showing a very weak expression (b); meanwhile, strong immunoreaction is seen in C5 (c), C7 (d), and C12 (e). Testosterone replacement (C12T group) restored the expression of both molecules (f), with staining pattern similar to S group. For negative controls, C12 sections were incubated with normal rabbit serum (g). Bar = 75 μm.

cells from orchiectomized rats showed nuclear localization of NF-κB (Fig. 6i). Finally, isolated mononuclear immune cells presented nuclear NF-κB expression in the prostate interstitium after androgen depletion (data not shown).

Orchiectomy up-regulated the expression of the antimicrobial molecules SP-D and rBD-1

Antimicrobial peptides, such as SP-D and defensins, are important components of the innate immune system against pathogens. Previous work from our laboratory demonstrated the presence of SP-D in the rodent prostate (Oberley *et al.*, 2007), supporting a similar finding in the human gland (Oberley *et al.*, 2005). Therefore, we wished to determine if SP-D protein expression is altered with androgen depletion. Orchiectomized rats exhibited a modest increase in this protein by 3 days after orchiectomy, with SP-D levels being highest at 7 days post-orchiectomy (Fig. 7a). Prostatic epithelial cells and secretion were the main sources of SP-D within the gland (Fig. 7c–e).

β-defensins are small peptides with strong antibacterial properties produced by various epithelial tissues such as the urogenital tract. We found very weak immunostaining for rBD-1 in the normal epithelium of the rat ventral prostate (Fig. 8b). However, the expression of this peptide was clearly augmented in orchiectomized rats in a time-dependent manner (Fig. 8a, and c–e). In addition, infiltrating immune cells after androgen deprivation were also positive for rBD-1 (Fig. 8d).

Apart from their direct antimicrobial abilities, SP-D and defensins have additional functions related to host defence, such as the induction of histamine release, cytokine increase and chemoattraction of immune cells. To further characterize the alterations in host defence proteins in the prostate post-orchiectomy, TNF-α expression was found to be increased in prostatic epithelial cells by 3–5 days, maintaining strong immunostaining at 7 and 12 days post-orchiectomy (Fig. 9a–c). Furthermore, these findings were correlated with multiple inflammatory signs, such as vasodilation and the presence of infiltrating CD3+ lymphocytes and $CD11^{b/c}$+ immune cells, which were readily apparent after orchiectomy (Fig. 9e–g and i–k), matching similar findings from Desai *et al.* (Desai *et al.*, 2004).

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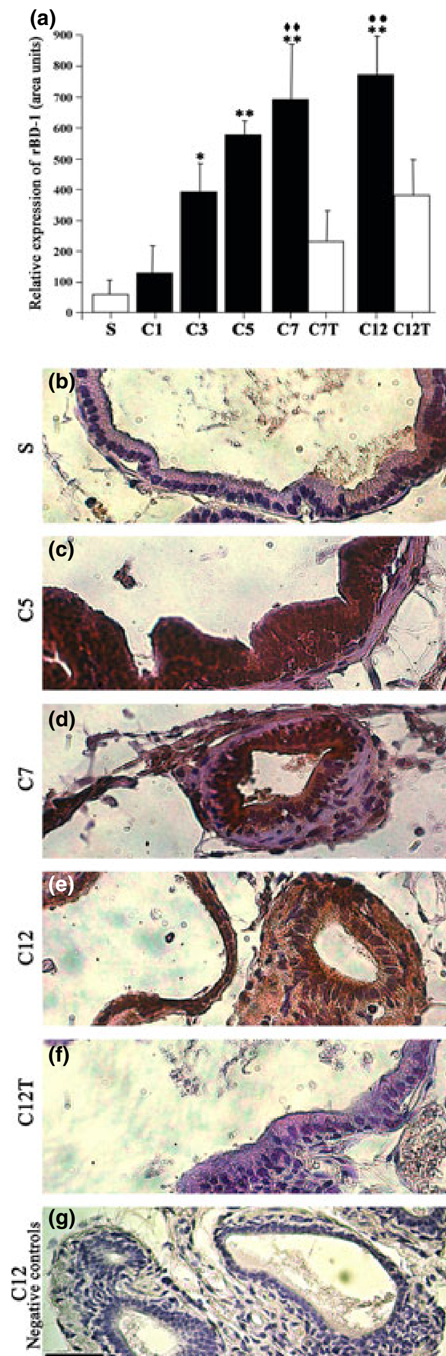

Figure 8 rBD-1 levels in rat prostate as determined by dot blot (a) and immunohistochemistry (b–g). Densitometric analysis (a) exhibiting an increase of rBD-1 in orchiectomized rats. Data were normalized with ACTB expression and represent the mean ± SE from at least three independent experiments. *vs. S $p < 0.05$, **vs. S $p < 0.01$, ♦♦ vs. C7T $p < 0.01$, •• vs. C12T $p < 0.01$ ANOVA -Tukey. The immunohistochemical examination shows scarce reactivity for rBD-1 in S group (b). In contrast, strong immunoreaction is seen in C5 (c), C7 (d), and C12 (e). Testosterone replacement (f) restored rBD-1, with a staining pattern similar to S group. For negative controls, C12 sections were incubating with normal rabbit serum (g). Bar = 75 μm.

Ex vivo and in vivo capacity of the prostate from orchiectomized animals to inhibit bacterial growth

Considering the increase in prostate host defence proteins after androgen ablation, we next evaluated the potential improvement in antibacterial ability of prostatic fluid from orchiectomized rats. A crude prostatic secretion preparation was first tested ex vivo for activity against the Gram-negative *E. coli* and the Gram-positive bacteria *S. aureus,* using the known ATB streptomycin and penicillin, respectively, as positive controls. Only prostate samples from rats at 5 days post-orchiectomy showed a slight inhibition zone (0.7 ± 0.1 mm, ANOVA -Tukey $p < 0.001$ C5 vs. C3, C7, C12, C7T, C12T and S) while streptomycin reached a mean of 6.6 ± 0.5 mm on *E. coli* cultures (Fig. 10a). All the remaining discs containing prostate fluid from the different experimental animals exhibited no inhibition zones in bacterial cultures of either *E. coli* or *S. aureus*.

To evaluate in vivo if orchiectomized prostates could eliminate bacterial pathogens more effectively than non-orchiectomized or orchiectomized and testosterone-replenished prostates, *E. coli* was inoculated into the ventral prostate. Rats were then killed at 5 days after infection to count bacterial growth within the gland. As shown in Fig. 10b, ventral prostates from orchiectomized animals (C3+INF, C5+INF and C7+INF) presented a lower count of CFU per mg of prostatic tissue compared with S+INF and C7T+INF. Furthermore, castrated rats exhibited few histological signs of prostatic inflammation when infected with *E. coli*, which was also correlated with weak immunostaining for *E. coli* antigens in the gland of this group (Fig. 10c).

Effect of testosterone on antibacterial activity of isolated prostatic cells in vitro

To verify in vitro direct effects of androgen deprivation on prostate host defences, prostatic cells were isolated and grown in media supplemented or not supplemented with testosterone. Under basal conditions, prostatic cells without testosterone tended to express more SP-D (Fig. 11a), with no differences found in the antibacterial activity against *E. coli* between supernatants from cells treated or not treated with testosterone (Fig. 11b). Bearing in mind that several immune modulatory effects are revealed after cellular activation, we applied the well-known bacterial compound LPS as a potent stimulus for cellular secretion of cytokines and antimicrobial substances.

As shown in Fig. 11, LPS-treated prostatic cells in the absence of testosterone increased significantly the expression of SP-D. To evaluate the putative improvement in

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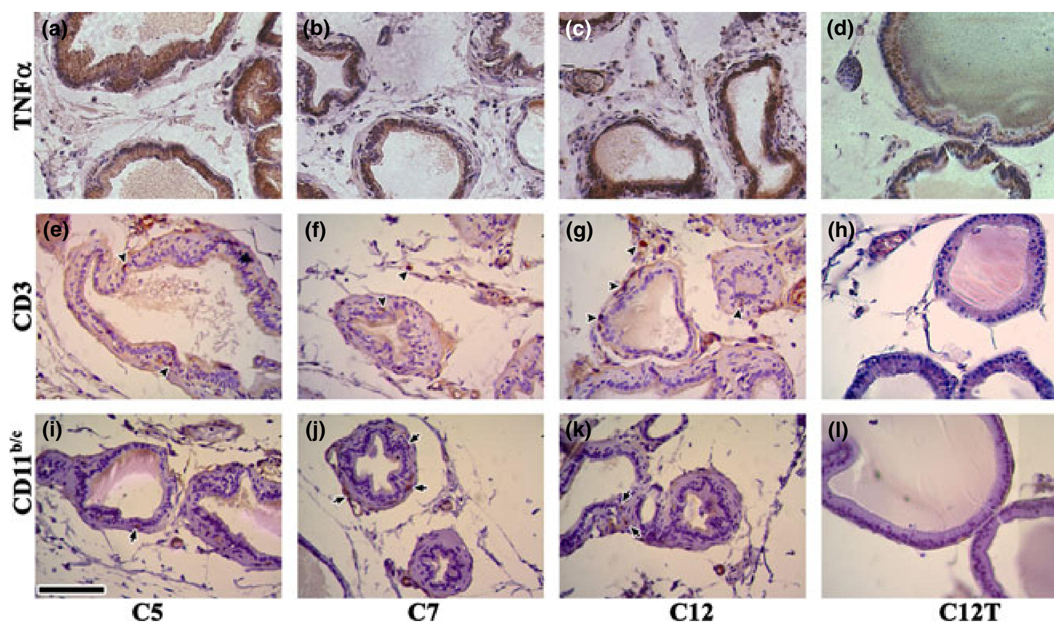

Figure 9 Presence of inflammatory signs in the prostate after orchiectomy. The regressing prostatic epithelium post-orchiectomy exhibits intense expression of the pro-inflammatory cytokine TNF-α (a–c). The immunocytochemical examination also reveals CD3+ cells, indicative of T lymphocytes, (arrowheads) and CD11 b/c+ cells (macrophages/dendritic cells, arrows) after 5 (e, i), 7 (f, j) and 12 days (g, k) after orchiectomy. Bar = 150 μm.

antibacterial activity related to the increment of SP-D in absence of testosterone, supernatants were incubated with *E. coli,* which resulted in a lower bacterial growth compared with supernatants from testosterone- and LPS-treated cells (Fig. 11b). As positive controls for bacterial killing, the ATB penicillin and streptomycin were added to the medium before the incubation with *E. coli*.

Discussion

The prostate is a strictly androgen-dependent organ which is the main target of infectious and inflammatory diseases in the male reproductive tract. The immunity of this gland has unique features which allowed considering the prostate as a site of immune privilege where responses are rather suppressed (Whitmore & Gittes, 1977). In the current study, we demonstrate that androgen withdrawal results in an increase of the pro-inflammatory TLR4 system and up-regulation of prostate antimicrobial host defences, correlating finally with an improved inhibition of bacterial growth in vivo as well as in vitro. Accordingly, testosterone might have a pivotal role in maintaining the gland in a basal immunosuppressive status.

Pathogen invasion of the body is largely prevented by physical barriers, immune cells and chemical compounds of the innate immune system, which acts as the first line of host defences (Zhao *et al.*, 1996; Palladino *et al.*, 2003; Avellar *et al.*, 2004; Kawai & Akira, 2010). Coincident with those on adaptive immunity, androgen immunosuppressive effects have also been reported on macrophages in vivo and in vitro (Miller & Hunt, 1996), where testosterone reduces the expression of the pathogen recognition receptor TLR4 (Rettew *et al.*, 2008) and pro-inflammatory products (D'Agostino *et al.*, 1999). In addition, androgen administration causes substantial reduction in NK cell activity in mice (Hou & Zheng, 1988). Moreover, sex differences in IFNα production by plasmocytoid dendritic cells after stimulation with HIV-1 single-stranded RNA have been described (Meier *et al.*, 2009), with enhanced CD8+T cell activation in women compared to men, explaining the clinical observation that women have a higher risk for HIV-1 disease progression during chronic infection at a given HIV-1 viral load (Farzadegan *et al.*, 1998). Together, these findings clearly contrast with the reported stimulatory effects of androgens on neutrophil activation after trauma and burn injuries, which make men more susceptible to acute shock aggressiveness than women (Deitch *et al.*, 2006).

In the male reproductive tract, the hormonal regulation of innate immunity gains special importance as a putative preventer of sperm damage as well as of venereal diseases. Epithelial cells lining the male tract express a wide range of host defence molecules, including TLRs, antimicrobial substances, immunomodulatory proteins and other mechanisms to eliminate microorganisms (Hall *et al.*, 2002;

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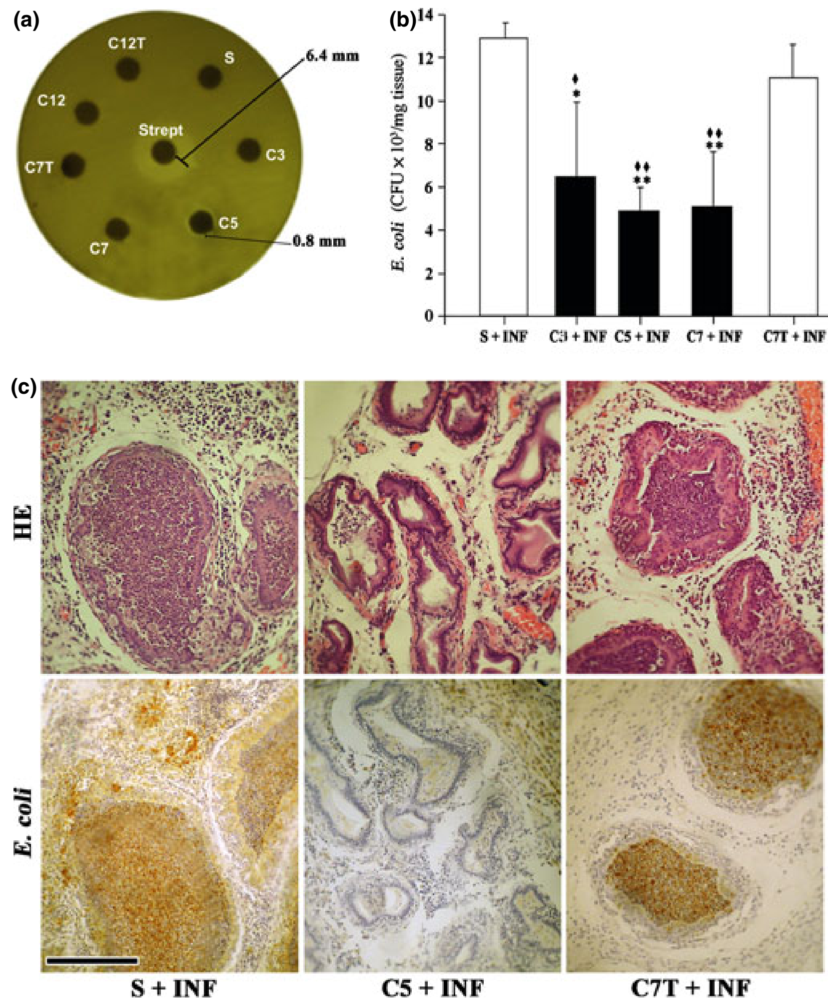

Figure 10 Effects of androgen depletion on the antibacterial activity of the prostate. Prostatic fluid from different time points of orchiectomy were tested ex vivo for activity against E. coli, with C5 exhibiting a slightly measurable inhibition zone (a) as compared to the positive control streptomycin (Strept) in a representative test. Bacterial counting indicates a better resolution of the infection in castrated animals (b) while panel C shows the histological features as well as the staining for *E. coli* present in S, C5 or C7T animals which were infected for 5 days. Data represent the mean ± SE from 10 rats per group in two independent protocols. *vs. S + INF $p < 0.05$, **vs. S + INF $p < 0.01$, ♦ vs. C7T + INF $p < 0.05$, ♦♦ vs. C7T + INF $p < 0.01$. Bar = 75 μm.

Palladino *et al.*, 2003, 2007; Girling & Hedger, 2007). The prostate gland is an important source of antimicrobial products such as zinc and lysozyme (Wichmann *et al.*, 1989; Hall *et al.*, 2002). Our results indicate that SP-D and rBD-1 also represent, although with a weak expression, host defences of the normal prostate, with their contents being incremental after orchiectomy. Even though the defensin family of antimicrobial peptides has been widely characterized in the epididymis (Hall *et al.*, 2002; Palladino *et al.*, 2003), its modulation by different stimuli is poorly documented in the male tract. In contrast to our findings in the prostate, the expression of rBD-1 mRNA in the initial segment and caput has been shown to decrease 15 days after orchiectomy, but was unchanged in the corpus and cauda epididymis (Palladino *et al.*, 2003). This discrepancy may be attributed to the trophic effects of androgens on male secretory products, with epididymal rBD-1 expression being repressed in orchiectomized animals. In contrast, the increased levels of prostatic rBD-1 and SP-D may be mediated by pro-inflammatory cytokines induced after orchiectomy. In agreement with this, we previously reported that androgen depletion leads to an increase of SP-D mRNA in the prostate of orchiectomized mice (Oberley *et al.*, 2007), where SP-D might be involved in removal of apoptotic cells (Stuart *et al.*, 2006). Nevertheless, orchiectomized rats exhibited better inhibition of bacterial growth, indicating that high levels of SP-D and rBD-1 may be responsible for the improved

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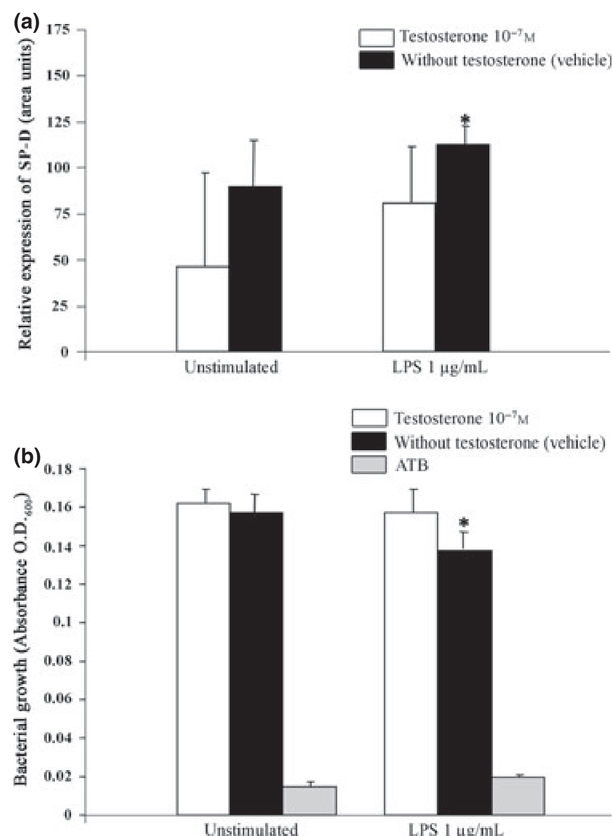

Figure 11 Antimicrobial properties of prostatic cells grown in presence or absence of testosterone in vitro. Densitometry of SP-D signals denoting a significant increase in cells without testosterone and stimulated with LPS (a). Values were normalized with ACTB expression and represent the mean ± SE from three independent experiments. Supernatants from prostatic cells were incubated with *E. coli* and analysed by turbidimetry (b). Antibiotics (ATB) were added to supernatants to be used as a positive control for bacterial inhibition. *$p < 0.01$ vs. LPS con testosterone.

antimicrobial activity herein reported. Indeed, the in vitro findings show that increased SP-D produced by isolated prostatic cells in absence of testosterone is associated to a better bacterial clearance.

We previously reported that both epithelial and stromal cells of the prostate are able to up-regulate pro-inflammatory signals after bacterial challenge in vivo (Quintar *et al.*, 2006, 2010) and in vitro (Gatti *et al.*, 2009; Leimgruber *et al.*, 2011), and these findings have been confirmed by several authors (Gatti *et al.*, 2006; Kundu *et al.*, 2008; Pei *et al.*, 2008). Our current results clearly indicate that testosterone negatively modulates the TLR4 pathway, including the expressions of TLR4, CD14 and MyD88 in prostatic cells. In line with this, androgens can inhibit the expression of TLR4 mRNA in human endothelial cells (Norata *et al.*, 2006) and can reduce TLR4 expression in the cell surface of isolated macrophages in mice (Rettew *et al.*, 2008). Furthermore, these results could explain, in part, the ability of testosterone to increase susceptibility to bacterial infection in both males and females (Rettew *et al.*, 2010), with castration being efficient to eliminate pathogens and to dampen infection-related inflammation within the prostate gland. Accordingly, androgen deprivation has been successfully employed as a therapeutic modality in rat (Kaplan *et al.*, 1983; Seo *et al.*, 2003) and canine (Cowan *et al.*, 1991) models of bacterial prostatitis.

The improvement in antimicrobial capacity of the prostate cells after androgen deprivation seems to be mainly independent of professional immune cells because in vitro, isolated prostatic cells in absence of testosterone reproduced, at least in part, the increase in host defence observed in vivo. This is a striking point because several effects of castration on prostatic cells do not represent direct effects of testosterone withdrawal but could be mediated by multiple cells infiltrating the gland after castration (Mercader *et al.*, 2001; Halin *et al.*, 2007). Interestingly, the effects of androgens on immunity could involve non-classical membrane androgen receptors which elicit rapid responses (Benten *et al.*, 2002, 2004). Nevertheless, studies that gain insight into the basis of the molecular mechanisms of testosterone affecting innate immunity are necessary.

The presence of testosterone appears to abrogate not only innate immunity but also adaptive responses within the prostate. There are several reports indicating that androgen ablation enhances prostate anti-tumour immunity (Roden *et al.*, 2004; Drake *et al.*, 2005; Koh *et al.*, 2009), even in castration-resistant tumours (Akins *et al.*, 2010). In addition, medical castration results in prominent T-cell infiltration of the human prostate (Mercader *et al.*, 2001) and removes tolerance to prostate cancer antigens in a transgenic mouse model (Drake *et al.*, 2005). Such T cell-mediated inflammation could have significant implications for the development of immunotherapeutic strategies to treat prostate cancer.

These data may represent an important mechanism underlying the immunomodulatory activity of testosterone in the prostate. However, this immunosuppressive function of androgens is understandable as a means of avoiding uncontrolled immune responses against the haploid male gamete in the reproductive tract.

The cellular effects of reproductive hormones on the male tract are thus complex and require a constant balance between response and tolerance to diverse antigens. Therefore, it would be too simplistic to ascribe a specific suppressive role to androgens on prostatic host defences. It is clear, however, that a better understanding of the inflammatory response and its regulation by

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androgens within the prostate gland may open new frontiers to develop efficient therapies for inflammatory and immune-related prostatic diseases based on homeostatic androgen functions.

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