Original Article

IL-27/IL-27RA signaling may modulate inflammation and progression of benign prostatic hyperplasia via suppressing the LPS/TLR4 pathway

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Background: Benign prostatic hyperplasia (BPH) is the most common urologic disease affecting aging men. The pathogenesis of BPH is multi-factorial, and chronic inflammation (CI) might be the central mechanism. Interleukin (IL)-27 signaling has been suggested as a modulator in autoimmune and inflammatory conditions. In this study, we used microarray experiments to analyze gene expression and molecular phenotypic associated with BPH progression, with a particular focus on CI and IL-27/IL-27RA signaling, and verified the microarray data in cell biology experiments.

Methods: Thirty BPH patients' specimens and clinical parameters were analyzed. BPH patients were divided into two groups based on the average prostate volume (41.5 mL): group 1, ≤40 mL; and group 2, >40 mL. Microarray experiments were conducted to identify differentially expressed genes (DEGs) by applying appropriate biostatistics to normalize and analyze the dataset. The candidate gene (*IL27RA*) was validated by quantitative reverse transcriptase-PCR (qRT-PCR) and immunohistochemistry (IHC). The interaction of *IL27RA* with genes involved in canonical inflammation-associated pathways was investigated by cell biology experiments.

Results: Eighty-three percent of BPH specimens contained inflammatory infiltrates, and the predominant type was CI. The serum PSA levels and prevalence of CI were higher in group 2. Microarray experiments identified 361 DEGs between these 2 groups. *IL27RA* was down-regulated and associated with prominent CI in BPH tissues of group 2. Validated by qRT-PCR and IHC, the results showed IL-27RA might modulate CI and progression of BPH. Thus, we investigated the interaction of *IL27RA* with *TLR4*, *IL6*, and *IL8*, which were involved in inflammation-associated pathways. We found the activation of IL-27RA after IL-27 treatment led to phosphorylation of STAT1 and STAT3 in prostate epithelial cells. By comparative treatments with lipopolysaccharide (LPS), IL-27, or combination, we found that IL-27/IL-27RA signaling suppressed the production of inflammatory cytokines, IL-6 and IL-8, induced by LPS/TLR4 pathway.

Conclusions: Our study revealed that down-regulation of *IL27RA* in prostate tissue was associated with higher prevalence of CI and BPH progression. IL-27/IL-27RA signaling suppressed the LPS/TLR4 pathway. We conclude the IL-27/IL-27RA signaling might modulate CI and provide potential therapeutic strategies to prevent BPH progression.

Introduction

Benign prostatic hyperplasia (BPH) is the most common urologic disease affecting aging men. The prevalence of BPH ranges from 50% to 75% among men 50 years of age and older, and up to 80% among men 70 years of age and older (1). BPH is histologically characterized by hyperplastic processes involving both the glandular and stromal compartments, forming nodules in the periurethral region and transition zone of the prostate, and leads to bladder outlet obstruction with various lower urinary tract symptoms (LUTS). Although symptomatic BPH is not usually life threatening, it can affect patients' quality of life, leading to morbidities and eventually causing a heavy burden on health care systems.

Age and hormones are well known contributing factors to the pathogenesis of BPH (2). However, the underlying molecular and cellular factors associated with BPH progression and LUTS are not fully understood (3). Many studies have suggested chronic inflammation (CI) could be a central mechanism in the development and progression of BPH (4-6). Several famous, large scale clinical studies, including the Medical Therapy of Prostatic Symptoms (MTOPS) study and the Reduction by Dutasteride of Prostate Cancer Event (REDUCE) trial, indicated that BPH patients with CI have been shown to have larger prostate volumes, more severe LUTS, and a higher probability of acute urine retention (AUR) than those without CI (7-9). Therefore, it has been hypothesized that prostatic inflammatory infiltration leads to tissue damage and to a chronic process of wound healing which activates hyperproliferative programs leading to hyperplastic nodules (10-13).

The etiology of chronic prostate inflammation is still unclear. Various potential sources exist for the initial instigating event, including direct infection, urine reflux inducing chemical and physical trauma, dietary factors, and estrogens (14). Among these, infection might be the main event for initiating a misguided immune response in the human prostate (15). König *et al.* (16) demonstrated pronounced expression of toll-like receptors (TLRs) and induction of immune-mediated inflammatory processes and cytokine secretion, e.g., interleukin (IL)-6, IL-8 and cyclooxygenase-2 (COX-2), in BPH tissues and prostate cancer (PCa) cells. Several reports also showed that lipopolysaccharide (LPS) stimulation could induce production of nitrite oxide (17), vascular endothelial growth factor and transforming growth factor β1 (TGFβ1) (18) in prostate epithelial cells. These findings suggest a key role of prostate epithelial cells in innate immunity, development of CI, tissue remodeling, and growth of BPH. Additionally, many mediators in CI have been investigated in BPH, including various growth factors and cytokines that have been shown to be involved both in the inflammatory process and in the interactions between epithelial and stromal prostate cells (5). These mediators are released by prostate cells and inflammatory cells (such as T lymphocytes) that can be found in most surgery-derived BPH specimens (19). Moreover, inflammatory mediators may contribute to growth of both prostate epithelial and stromal cells directly through cytokines that stimulate the production of prostate growth factors and indirectly through decreases in prostate cell death via down-regulation of apoptosis (20,21).

IL-27 is a heterodimeric cytokine composed of the subunit protein IL-27p28 and Epstein Bar virus-induced gene 3 (EBI3) (22). IL-27 binds to a heterodimeric receptor complex, which is composed of a ligand-binding chain, IL-27 receptor, α subunit (IL-27RA, also known as WSX-1 or TCCR), and an additional signal-transducing chain, gp130 (23). IL-27 activates Janus kinase (JAK) and signal transducer and activator of transcription 1 (STAT1) and STAT3, which modulate the differentiation of T help cell (Th) types through the activities of downstream transcription factors, such as T-bet (Th1) (24), GATA-3 (Th2) (25) and retinoic acid receptor-related orphan receptor γt (Th17) (26), leading to commitment of Th1 differentiation, Th2/Th17 suppression and IL-10 induction. Through these mechanisms, IL-27 signaling plays an immunosuppressive role and suppresses production of proinflammatory cytokines (27,28). To date, the anti-inflammatory properties of IL-27 have only been described in T cells, and so the effect of IL-27 signaling on prostate

epithelium is unclear.

The distribution of the IL-27 receptor is not restricted to immune cells; it is also expressed in endothelial cells, keratinocytes, and in mammary, lung, and prostate epithelium (23). Recent studies have reported the role of IL-27 in PCa, where it was shown to reduce invasive potential in vitro, including cell viability and migration (29), and to reduce proliferation and vascularization in xenograft tumors (30). Moreover, in human prostate tissues, IL-27RA was found to be expressed by normal epithelia and low grade PCa, but not in tumors of high grade or advanced stage (30). Collectively, these findings suggest that IL-27 signaling might directly suppress PCa growth *in vitro* and in vivo. However, the role of IL-27/IL-27RA signaling in CI and BPH progression is still unclear.

Considering the importance of CI in the development and progression of BPH, we tried to find potential biomarkers/genes associated with prostatic inflammation and growth through gene expression analysis, and to validate them. We first collected prostate tissues from BPH patients, and characterized their inflammatory status and clinical parameters. Then, we conducted a genome-wide microarray analysis of BPH tissues and compared gene expression profiles in subgroups of patients with different prostate volumes (group 1, ≤40 mL; group 2, >40 mL). *IL27RA* was significantly down-regulated in group 2 compared with group 1. The expression of *IL27RA* in prostate tissue was validated by quantitative reverse transcriptase-PCR (qRT-PCR) and immunohistochemistry (IHC). Thus, we hypothesized that *IL27RA* might play a role in modulating prostatic inflammation and BPH progression. Furthermore, based on pathway analysis, we performed cell biology experiments to validate *IL27RA* and its interaction with several genes including *TLR4*, *IL6*, and *IL8*, which were simultaneously up-regulated in canonical pathways associated with immune/inflammatory responses. The cell biology experiments suggested that IL-27/IL-27RA signaling might play the role of immune regulator in prostate epithelial cells, and offer a novel therapeutic strategy for attenuating prostatic CI and halting progression of BPH.

Methods

Collection of patients' data and samples, ethics statement, and grouping

Thirty prostate specimens were obtained from patients

who underwent the transurethral resection of the prostate (TURP) and transrectal ultrasound (TRUS)-guided needle biopsy of the prostate at the Division of Urologic Surgery, Department of Surgery, Songshan Branch of Tri-Service General Hospital (TSGH) from 2007 to 2009. The tissue samples from prostate biopsies were consistently taken from the transitional zones (TZ) area after suspicious malignancy (either in TZ or peripheral zones) were excluded preliminarily by TRUS. Histological diagnosis of BPH was confirmed by an independent pathologist. Either the specimens from TURP or biopsy, the patients whose pathologic reports showed cancerous or precancerous lesions were excluded from this study. Prostate tissue specimens were immediately immersed in RNA*later* buffer (Applied Biosystems), snap-frozen in liquid N_2, and stored at –80 ℃ for RNA extraction. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board of TSGH (TSGHIRB Approval Number: 096-05-177) and an informed consent was taken from all the patients. BPH patients were divided into two groups based on the average BPH volume (41.5 mL): group 1, prostate volume ≤40 mL; and group 2, prostate volume >40 mL.

Isolation and labeling of total RNA for gene expression profiling

Total RNA from the tissue specimens was isolated using TRIzol reagent (Invitrogen) and purified with an RNeasy mini kit (Qiagen) according to the manufacturers' instructions. Labeled cRNA was synthesized using purified total RNA (1–15 µg) as a template following Illumina's standard synthesis protocols. HumanRef-8 v2 Expression BeadChip 2.0 arrays (Illumina, Inc.) were hybridized to the biotinylated cRNA targets. After 16 hours of hybridization at 45 ℃, the arrays were washed by a Fluidics Station 450 and scanned by a GeneChip Scanner 3000.

Data mining and pathway analysis

After scanning, the intensity data from the GeneChip were analyzed by Partek Software (Partek Genomics Suite, RRID:SCR_011860, Partek Inc., St. Louis, MO, USA) for mRNA expression levels. We used the Partek Software to conduct robust multi-array average analysis, which contains background correction, quantile normalization, probe level intensity calculation, and significant gene identification. After data processed, Partek Software was also used to

conduct principal component analysis (PCA), which reduces higher-dimensional data into a three-dimensional graph, and to evaluate the similarity of gene expression profiles for different samples. Then we used the Genesis program (RRID:SCR_015775), which is a platform to simultaneously visualize and analyze a whole set of gene expression data, to perform hierarchical clustering (HC) analysis and generate a visual representation (heatmap) of the expression profiles. Finally, Ingenuity Pathway Analysis (IPA, RRID:SCR_008653) was applied to describe gene-gene interaction networks, biological functions, and canonical pathways of differentially expressed genes.

Quantitative reverse transcriptase-PCR

After reverse transcription of 1μg total RNA, real-time PCR was performed by ABI 7300 (Applied Biosystems) with SYBR Green (Sigma) according to standard protocols. The PCR primers were as follows: forward primer *IL27RA*, 5'-TGCAGGTGAGCTACAAAGTCTGGT-3', reverse primer *IL27RA*, 5'-AGCAGACCAAAGAGAGGTTGGTGA-3'. Each measurement was made in triplicate and normalized to an 18s RNA (forward primer, 5'-TCAACTTT CGATGATGGTAGTCGCC-3', reverse primer, 5'-TCCTTGGATGTGGTAGCCGTTT-3') control to ensure comparable amounts of cDNA in all wells.

Immunohistochemical staining

Tissue sections (4-μm thick) were constructed from paraffin-embedded tissues and used for immunostaining. The tissue sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. The tissue sections were then boiled in citrate buffer (pH 9) for 10 min and treated with 3% hydrogen peroxidase to block endogenous peroxidase activity. After washing in phosphate-buffered saline, the tissue sections were incubated with normal goat serum (dilution 1:500; Dako) for 30 min and incubated with IL-27RA antibody (dilution 1:50; Novus) overnight at 4 ℃. Then biotin- and streptavidin-labeled antibodies were used for 3,3'-diaminobenzidine (DAB) staining. Prostate epithelial cells showing cytoplasmic immunoreactivity to IL-27RA antibody were scored as having positive IL-27RA expression.

Chemicals and antibodies

Lipopolysaccharide (LPS; from *Escherichia coli* 0127:B8) was purchased from Sigma-Aldrich. Recombinant human cytokine IL-27 was purchased from R&D Systems. Antibody against IL-27RA (Novus Cat# NBP1-02708, RRID:AB_2125066) was purchased from Novus. Anti-phospho-STAT1(Y701) (Cell Signaling Technology Cat# 9171, RRID:AB_331591), anti-phospho-STAT3(Y705) (Cell Signaling Technology Cat# 4113, RRID:AB_2198588), anti-STAT1 (Cell Signaling Technology Cat# 9172, RRID:AB_2198300) , and anti-STAT3 (Cell Signaling Technology Cat# 9132, RRID:AB_331588) antibodies were purchased from Cell Signaling Technology. Anti-TLR4 (Abcam Cat# ab13556, RRID:AB_300457), anti-IL-6 (Abcam Cat# 1457-1, RRID:AB_562150), and anti-IL-8 (Abcam Cat# ab34100, RRID:AB_775629) antibodies were purchased from Abcam. Beta(β)-actin (Millipore Cat# MABT825, RRID:AB_2571580) and horseradish peroxidase linked anti-rabbit (Millipore Cat# AP307P, RRID:AB_92641), anti-mouse (Millipore Cat# AP308P, RRID:AB_92635) and anti-goat (Millipore Cat# AP106P, RRID:AB_92411) secondary antibodies were purchased from Millipore. Secondary antibodies for IHC were from Dako.

Cell culture and treatment

Human PCa derived epithelial cell lines PC-3 (BCRC, Cat# 60122, RRID:CVCL_0035), DU-145 (BCRC, Cat# 60348, RRID:CVCL_0105), and LNCaP (BCRC, Cat# 60088, RRID:CVCL_1379) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The BPH epithelial cell line BPH-1 was a kind gift provided by Simon W. Hayward (Vanderbilt University Medical Center, Nashville, TN, USA). Normal human prostate stroma cell line WPMY-1 (ATCC, Cat# CRL-2854, RRID:CVCL_3814) and normal epithelial cell line RWPE-1(ATCC, Cat# CRL-11609, RRID:CVCL_3791) were purchased from the American Type Cell Collection. WPMY-1 cells were grown in Roswell Park Memorial Institute (RPMI) medium containing 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (Invitrogen) in a humidified 5% CO_2 atmosphere at 37 ℃, whereas RWPE-1 cells were cultured in keratinocyte-serum free medium supplemented with recombinant human epidermal growth factor and bovine pituitary extract.

The effect of IL-27 signaling on BPH-1 and PC-3 cells was evaluated. The cells were treated with IL-27 at various concentrations (0, 50, 100 ng/mL) and incubated for 30 min (dose–response assay). For the time–response assay, the cells were treated with 100 ng/mL of IL-27 for different

times: 0, 30, and 60min. Stimulated and non-stimulated cells from those cultures were used to observe the total and phosphorylated status of STAT1 and STAT3, respectively.

To determine the response to LPS stimulation on prostate cell lines, the BPH-1 and PC-3 cells were treated with LPS (10 µg/mL) (17,18) and incubated for different times: 0, 3, 6, 12, and 24 h. Stimulated and non-stimulated cells as well as supernatants from those cultures were collected in different experiments.

To determine the effects of IL-27 signaling on activation of the TLR4 pathway induced by LPS, BPH-1 and PC-3 cells were incubated with 100 ng/mL IL-27 at least 30 min before LPS 10 (µg/mL) was added and further incubated for 6 h. Non-stimulated cells were also treated with IL-27 at the same concentrations as the LPS-stimulated cells. Stimulated and non-stimulated cells and supernatants from the cell cultures were collected for the analyses described below.

Western blotting

Fifty to one hundred µg of total cellular protein were separated by 10% SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes. The membrane was blotted with the primary antibody overnight at 4 ℃ and with the appropriate secondary antibody for 1 h at room temperature the next day. Visualization was performed by enhanced chemiluminescence (Millipore) using a UVP BioSpectrum AC Imaging System. To semiquantitate and compare the protein levels, the gels were photographed and the intensities of the bands were analyzed using ImageJ software (ImageJ, RRID:SCR_003070). The relative band intensities in each reaction were normalized to the mean intensity of the β-actin band.

Enzyme-linked immunosorbent assay (ELISA) analysis

Supernatants were collected and centrifuged at 12,000×g for 15 min to remove cellular debris. A commercial ELISA reaction kit (eBioscience) for IL-6 and IL-8 release from cell supernatants was used according to the manufacturer's protocol.

Statistical analysis

Clinical parameters were compared between the prostate sample groups which were divided by mean prostate volume. A χ^2 test was used to compare qualitative data between the groups. For comparison of quantitative data, a Mann-Whitney test was used.

We used t-test method to reveal target genes. According to mean values, the gene probes whose "fold change" value showed at least >1.4 or <–1.4 expression difference were enrolled into study. To ensure high reliability of the fold change values obtained from the probes, we set the "false discovery rate" (FDR) <25%, and the FDR corrected P<0.05 were considered significant. The FDR was determined by using the Benjamini-Hochberg multiple testing procedure.

In cell biological experiments, all data were representative of more than three independent experiments, with each performed in triplicate. Levels of significance between samples were determined by the Student's t-test. A value of P<0.05 was considered statistically significant.

Results

Clinical characteristics of BPH patients

Patient characteristics are presented in *Table 1*. The mean age of BPH patients enrolled in this study was 73.4±9.9 years. Tissue samples were obtained from 5 patients with elevated PSA via TRUS-guided needle biopsy, and 25 patients with or without elevated PSA underwent TURP for symptomatic BPH. The mean prostate volume measured by TRUS was 41.5±21.1 mL (range 12 to 85 mL). The average serum PSA level was 13.2 ng/mL (range 1.3 to 46.5 ng/mL). Most of the specimens revealed inflammatory infiltration (83.3%), and of these, the percentages of acute, chronic, and mixed acute and chronic inflammation were 8%, 64% and 28%, respectively. BPH patients were divided into two groups based on prostate volume as described in the Methods. Several clinical parameters were compared between these 2 groups. The preoperative prostate volume and PSA level were significant between them, whereas the IPSS, maximal uroflow rate, post void residual volume, and surgery type were insignificant. The prevalence of inflammation in each group was similar and showed no statistical difference (85.7% *vs.* 81.3%). However, the serum PSA levels and prevalence of CI were significantly higher in group 2 compared with group 1 (PSA: 15.8 *vs.* 10.5; CI: 62.5% *vs.* 42.8%). These data suggest that CI is positively correlated with PSA level and prostate volume, as well as BPH progression.

Gene expression profiling in BPH tissues

Of the 30 patients, 23 samples (10 from group 1 and 13

Table 1 Patients' demography and comparison of clinical parameters and histological characteristics

	All patients (n=30)	Group 1 (n=14)	Group 2 (n=16)	Statistical Analysis, P value
Average age (SD) (range, median, IQR)	73.4 (9.9) (47–88, 75.5, 13)	76.2 (9.4) (47–81, 78, 6)	70.6 (9.9) (57–88, 72, 18)	Mann-Whitney, 0.089
Average preoperative prostate volume (mL) (SD) (range, median, IQR)	41.5 (21.1) (12–85, 40, 30)	22.2 (11.7) (12–40, 20, 23.1)	55.8 (15.3) (41.8–85, 50, 30.2)	Mann-Whitney, <0.01
Average preoperative serum PSA value (ng/mL) (SD) (range, median, IQR)	13.2 (15.9) (1.3–46.5, 7.3, 10.2)	10.5 (17.6) (1.3–42.2, 4.9, 4.5)	15.8 (14.1) (2.2–46.5, 11.4, 9.2)	Mann-Whitney, <0.05
Average preoperative IPSS (SD) (range, median, IQR)	28.6 (6.5) (13–35, 30.5, 5)	29.1 (6.4) (13–35, 30.5, 5)	28.1 (6.8) (14–35, 30.5, 5)	Mann-Whitney, 0.674
Average preoperative Qmax (mL/min) (SD) (range, median, IQR)	8.9 (2.7) (4.8–14, 8.9, 3.3)	8.7 (2.4) (4.8–13.2, 8.5, 3.1)	9.1 (3.0) (3.9–14, 9.3, 4.2)	Mann-Whitney, 0.631
Average preoperative post void residual volume (mL) (SD) (range, median, IQR)	87.6 (62.7) (0–250, 80, 81)	84.1 (58.6) (0–200, 77.5, 81)	90.7 (67.9) (5–250, 85, 87.5)	Mann-Whitney, 0.904
Surgery type: biopsy/TURP	5/25	2/12	3/13	χ^2 test, 0.743
Inflammation (presence/absence) (acute/chronic/mixed/absence)	83.3% (25/5) (2/16/7/5)	85.7% (12/2) (0/6/6/2)	81.3% (13/3) (2/10/1/3)	χ^2 test, 0.743
IL-27RA staining positive: negative (% of positive stain)	11: 19 (36.6%)	8: 6 (57.1%)	3: 13 (18.7%)	χ^2 test, <0.05

SD, standard deviation; IQR, interquartile range; PSA, prostatic specific antigen; IPSS, international prostate symptom score; Qmax, maximal uroflow rate; TURP, transurethral resection of prostate; IL-27RA, interleukin-27 receptor, alpha subunit.

from group 2) with good RNA quality were subjected to microarray experiments. The gene expression of BPH tissue in group 1 versus group 2 was analyzed using the high-throughput whole genome BeadChip arrays. We found 361 differentially expressed genes with a fold change of >1.4 or <–1.4. Among them, 214 genes (59.2%) were down-regulated and 147 genes (40.8%) were up-regulated in BPH tissue of group 2 compared with group 1. PCA was used to examine whether the differentially expressed genes could be used to distinguish larger volume from smaller volume BPH tissues. The results of PCA showed that larger volume (group 2) BPH tissues aggregated to the upper right side, whereas smaller volume (group 1) BPH tissues clustered to the lower left, indicating that the differentially expressed genes could be used to separate the tissue samples into two distinct groups (*Figure 1A*). HC analysis showed different patterns of gene expression between the 2 groups of BPH patients (*Figure 1B*).

Differential expression of immune-inflammation signaling pathways between two groups of BPH patients

To investigate which genetic networks and canonical pathways were significantly changed in group 2 compared with group 1, IPA was performed. The functions of "Protein Synthesis, Gene Expression, RNA Trafficking", "Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair", and "Cell Signaling, Gene Expression, Cell Cycle" were identified as the major functions of the top three genetic networks (*Table S1*). We also identified 10 canonical pathways that were significantly [–log (P) >4.27] enriched in larger volume BPH tissues (*Table S2*). The top three pathways with the most significant P value were "Triggering receptor expressed on myeloid cells 1" (TREM1) signaling, "Liver X receptor/retinoid X receptor" (LXR/RXR) signaling, and "Communication between innate and adaptive immune cells". Upon Inspection of genes listed in the
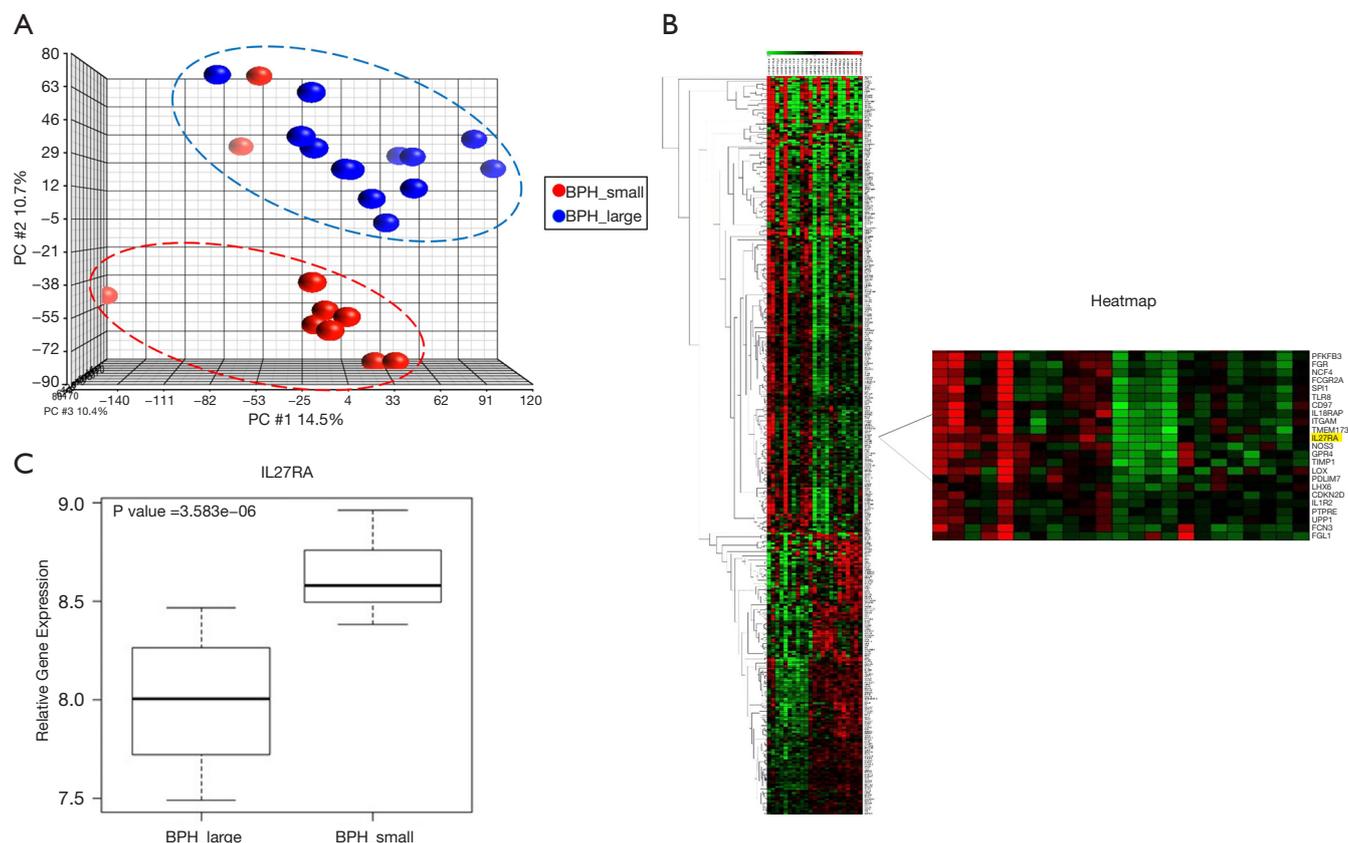
Figure 1 Microarray analysis of gene expression in benign prostatic hyperplasia (BPH) tissues. (A) Principal component analysis of the BPH samples (n=23). Red dots denote small volume BPH tissues; blue dots denote large volume BPH tissues. Each dot represents the expression values of the significant genes that were summarized on the first two principal component coordinates. (B) Hierarchical clustering of the 361 significantly different genes. Input data are the log2 ratios of large BPH intensity over small BPH intensity. Red indicates genes that were upregulated in BPH tissues; green indicates down-regulated genes. The data are organized by prostate size: labels of small samples are in black (n=10, left side) and those of large samples in orange/gold (n=13, right side). (C) A box plot shows the relative expression of *IL27RA* between large and small volume BPH tissues by quantitative reverse transcriptase-PCR (qRT-PCR). 18s RNA was used for an internal control.

above pathways, we identified several genes that were up-regulated simultaneously, including *IL6*, *TLR4*, and *IL8* (*Figure S1*), which might participate in promoting prostatic inflammation and progression of BPH.

Down-regulation of IL27RA in BPH tissue of group 2

The IPA showed that the pathways associated with immune/inflammation regulation were significantly different between two groups of BPH patients. To identify potential biomarkers or candidate genes involved in CI that might lead to BPH, we focused on genes associated with immune/inflammation response and modulation, and found that

IL27RA was significantly down-regulated in group 2. To validate the results of the microarray analysis, qRT-PCR was conducted in each of the 30 BPH specimens. As shown in *Figure 1C*, qRT-PCR results were consistent with the microarray data, and *IL-27RA* expression was significantly (P=3.583E-06) down-regulated in group 2. Additionally, IHC stains were used to validate the expression of IL-27RA in BPH tissue. Positive expression of IL-27RA was defined as being immunoreactive to IL-27RA antibody in the glandular epithelial cells of prostate tissue (*Figure 2A*). The results showed less positive expression of IL-27RA in group 2 compared with group 1 (18.7% *vs.* 57.1%; P<0.05, *Table 1*), suggesting that less expression of IL-27RA in BPH

epithelium might be associated with more CI and increased progression of BPH.

Constitutive expression of IL-27RA and TLR4 in human prostate cells

Motivated by the above results showing that several genes involved in immune/inflammation associated canonical pathways were simultaneously up-regulated, while the expression of *IL27RA* was significantly down-regulated in group 2, we hypothesized that *IL27RA* might modulate CI and BPH progression via regulating the known *TLR4* pathway and its downstream products, *IL6* and *IL8* (16). Western blotting was performed to verify the presence of IL-27RA and TLR4 in prostate cell lines, and as shown in *Figure 2*, IL-27RA and TLR4 were differentially expressed in epithelial cells (RWPE-1, BPH-1, PC-3, DU-145) and in stroma cells (WPMY-1). The expression of IL-27RA was highest in RWPE-1, BPH-1 and PC-3 cells (*Figure 2B*), while TLR4 expression was highest in BPH-1, DU-145, and PC-3 cells (*Figure 2C*). The higher level of TLR4 expression in hyperplastic BPH-1 cells and in PCa-derived DU-145 and PC-3 cells in comparison to the normal epithelial cells, RWPE-1, suggests that the TLR4 pathway might play a connecting role between inflammation and prostate cell proliferation in BPH or carcinogenesis in PCa.

LPS-induced IL-6 and IL-8 expression in BPH-1 and PC-3 cells

The fact that TLR4 is constitutively expressed in prostate epithelial cells indicates that either microbial or endogenous materials could trigger inflammatory responses in these cells (16). Given the findings above from microarray and western blot analyses, we hypothesized that TLR4 signaling could induce production of proinflammatory cytokines, IL-6 and IL-8, in BPH-1 and PC-3 cells by LPS stimulation. Thus, a time-course study was conducted, and western blotting and ELISA were used to characterize the expression levels of IL-6 and IL-8 at 0, 3, 6, 12, and 24 h after LPS exposure. In BPH-1 cells, we found that IL-6 was significantly increased at 6 h after LPS stimulation in western blotting (*Figure 3A*) and ELISA (*Figure 3B*), whereas IL-8 was progressively elevated with time in the ELISA, but undetectable in western blotting. In contrast, the levels of IL-6 and IL-8 followed the same pattern of expression over time upon LPS stimulation in PC-3 cells, reaching a peak at 6 h in

both western blotting (*Figure 3C*) and ELISA analyses (*Figure 3D*). These results showed that both BPH-1 and PC-3 cells can activate innate immunity and initiate an inflammatory response to LPS by inducing production of IL-6 and IL-8 in a time-dependent manner.

Suppression of LPS-induced IL-6 and IL-8 expression by IL-27

The induction of IL-6 and IL-8 in prostate tissues has been shown to be not only associated with the inflammatory response in BPH, but involved in proliferation, malignant transformation, and migration in PCa (31,32). Our data have shown the presence of IL-27RA and of IL-27/IL-27RA signaling. However, the functions of IL-27/IL-27RA signaling in prostate epithelial cells have not been studied. Thus, we used conditional treatment to investigate the effects of IL-27 signaling on LPS-induced expression of IL-6 and IL-8 in BPH-1 and PC-3 cells. Each prostate cell line was treated with either LPS (10 μg/mL) only, IL-27 (50 ng/mL) only, or combined LPS (10 μg/mL) and IL-27 (50 ng/mL). In BPH-1 cells, we found that treatment with IL-27 suppressed on LPS-induced expression of both IL-6 and IL-8 (*Figure 4A,B*). IL-27 treatment alone had insubstantial effects on the expression of IL-6 and IL-8. Similar results were observed in PC-3 cells (*Figure 4C,D*). Collectively, our findings suggest that IL-27/IL-27RA signaling can suppress the expression of inflammatory cytokines, IL-6 and IL-8, induced by LPS stimulation.

Activation of IL-27/IL-27RA signaling leads to phosphorylation of STAT1 and STAT3

We have showed that there is constitutive expression of IL-27RA in prostate cells (*Figure 2B*), but the role and mechanism of IL-27/IL-27RA signaling in BPH-1 and PC-3 cells is still unclear. IL-27 has been described in lymphocytes to activate both the STAT1 and STAT3 pathways (33). By western blotting, we found that phosphorylation of STAT1 and STAT3 were increased in BPH-1 and PC-3 cells by IL-27 stimulation in a dose-dependent manner (*Figure 5*, right panels). In time-response experiments, the phosphorylation of STAT1 and STAT3 were found to be induced at 30 and 60 min after IL-27 stimulation (*Figure 5*, left panels). These results showed that IL-27RA signaling in BPH-1 and PC-3 cells leads to phosphorylation of STAT1 and STAT3 after IL-27 stimulation.
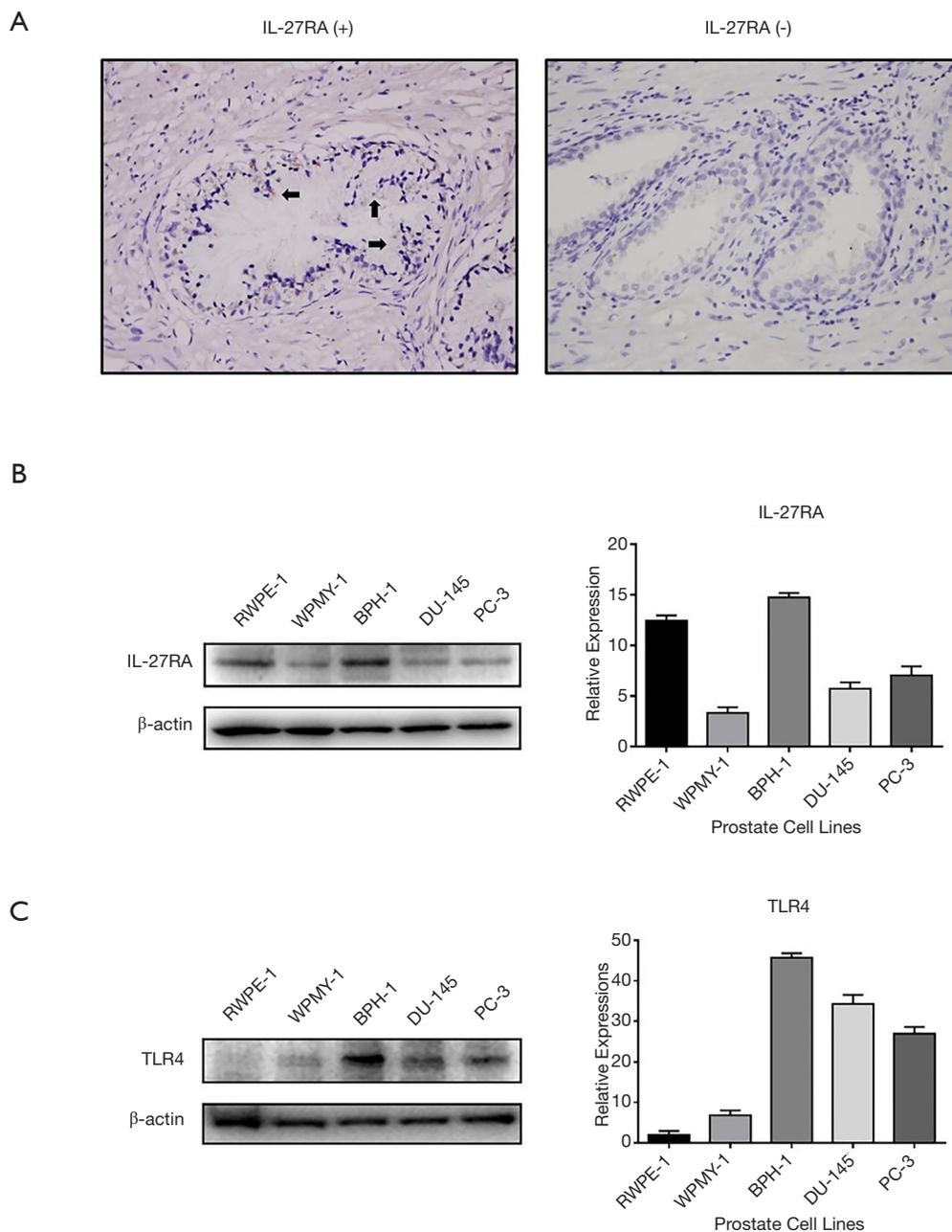
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Figure 2 Immunohistochemical validation of Interleukin-27 receptor, α subunit (IL-27RA) expression in BPH specimens and relative expression of toll-like receptor 4 (TLR4) and IL-27RA in the human prostate cell lines. (A) Representative images of immunohistochemical staining for IL-27RA protein on primary specimens from BPH patients. Patients were divided into the positive IL-27RA group (left) or the negative IL-27RA group (right), with sections of nodular gland showing immunoreactivity to IL-27RA antibody (arrow) on the surface of prostate epithelial cells (original magnification: 200×). The relative expression of IL-27RA (B) and TLR4 (C) was determined by western blotting in prostate cell lines RWPE-1, WPMY-1, BPH-1, DU-145, and PC-3. Data was representative of more than three independent experiments, with each performed in triplicate. Semi-quantitation was done by densitometry using ImageJ software.
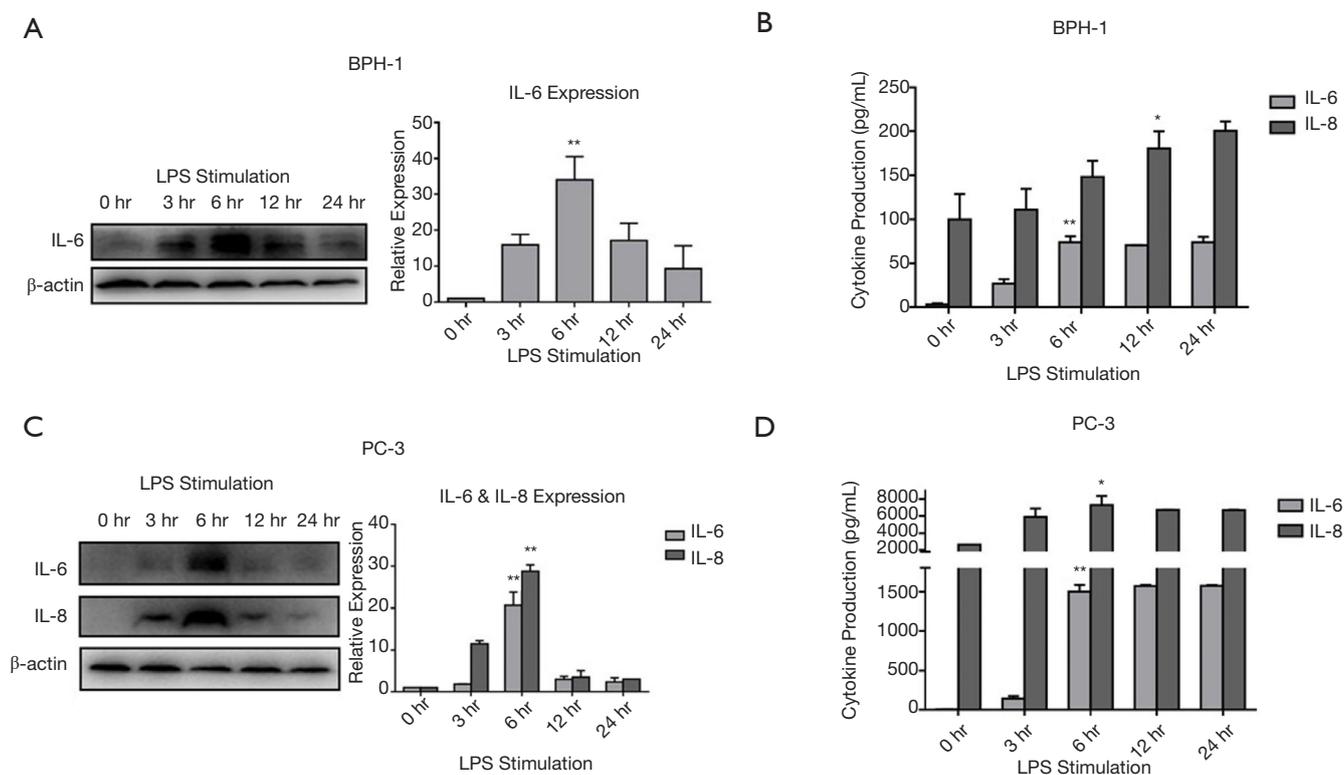
Figure 3 The relative expression of proinflammatory cytokines, interleukin 6 (IL-6) and IL-8, in BPH-1 and PC-3 prostatic cells after Lipopolysaccharide (LPS) stimulation. BPH-1 and PC-3 cells were treated with LPS (10 µg/mL) for 0, 6, 12, and 24 h, and then cellular proteins and cell-free culture supernatants were collected for western blotting and ELISA analysis, respectively. The relative expression of IL-6 and IL-8 in BPH-1 cells after LPS stimulation at different time points was determined by western blotting (A) and enzyme-linked immunosorbent assay (ELISA) analysis (B). The western blotting showed the expression of IL-8 was absent in BPH-1 cells. The relative expression of IL-6 and IL-8 in PC-3 cells after LPS stimulation at different time points was measured by western blotting (C) and ELISA analysis (D). Semi-quantitation of western blotting was done by densitometry using ImageJ software. Data was representative of more than three independent experiments, with each performed in triplicate. *P<0.05, **P<0.001.

Discussion

To our knowledge, this is the first study of its kind to specifically investigate gene expression focusing on CI and BPH progression. We first assessed the characteristics of a group of Taiwanese BPH patients, and analyzed the prevalence and patterns of inflammation in their specimens. These specimens were characterized by indicators of CI. Microarray experiments identified *IL27RA* as a potential genetic biomarker significantly down-regulated in BPH. In vitro experiments were performed to investigate IL-27/IL-27RA signaling and its interaction with several genes, such as *TLR4*, *IL6*, and *IL8*, which were identified from IPA. Our results showed that IL-27/IL-27RA signaling could modulate the inflammatory response induced by LPS/TLR4 signaling in BPH-1 and PC-3 cells. Collectively,

these data support a novel role of IL-27/IL-27RA signaling in modulating CI and progression of BPH.

In our clinical data (*Table 1*), we found that prostatic inflammation was widely distributed in BPH specimens, and that among subtypes of inflammation, CI positively correlated with BPH volume. Although the role of CI in BPH pathogenesis is still unclear, several studies have provided supportive evidences. These studies concluded that patients with prostatic inflammation were more likely to progress clinically in terms of worse symptoms, AUR or BPH related surgery (8,9). A significant association among the degree of prostatic inflammation, prostate volume, and serum PSA levels was confirmed (7,19). Consistently, we found that there were higher serum PSA levels and a higher percentage of CI in larger volume prostate tissues compared
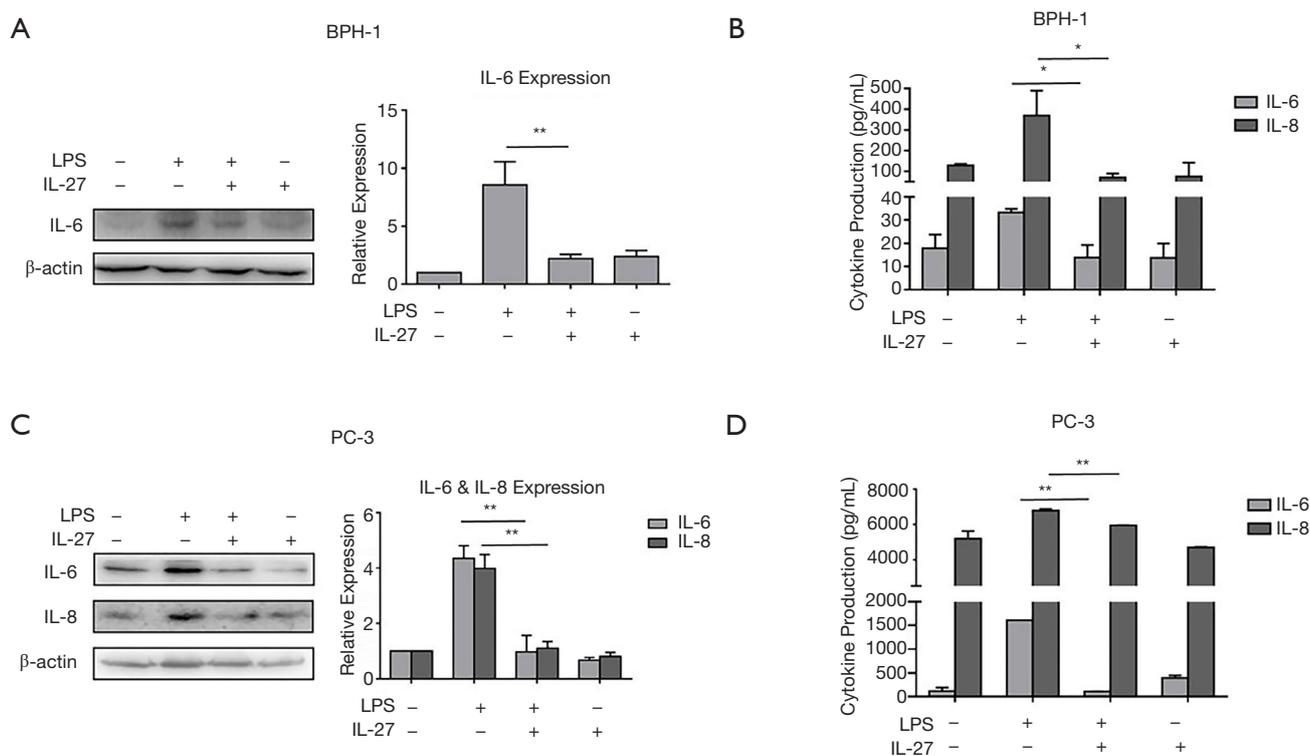
Figure 4 Suppression of Lipopolysaccharide (LPS)-induced interleukin 6 (IL-6) and IL-8 expression by IL-27/IL-27 receptor, α subunit (IL-27RA) signaling in BPH-1 and PC-3 cells. BPH-1 and PC-3 cells were treated with culture medium, LPS (10 µg/mL), LPS (10 µg/mL) plus IL-27 (50 ng/mL), and IL-27 (50 ng/mL) for 6 h. Then the cellular proteins and cell-free supernatants were collected and evaluated by western blotting and ELISA analysis. The relative expression of IL-6 and IL-8 in BPH-1 cells after treatment with LPS and/or IL-27 was determined by western blotting (A) and enzyme-linked immunosorbent assay (ELISA) analysis (B). The western blotting showed the expression of IL-8 was absent in BPH-1 cells. The relative expression of IL-6 and IL-8 in PC-3 cells after treatment with LPS and/or IL-27 was measured by western blotting (C) and ELISA analysis (D). IL-6 and IL-8 expression in the group treated with LPS plus IL-27 was compared with group treated with LPS only. Semi-quantitation of western blotting was done by densitometry using ImageJ software. Data was representative of more than three independent experiments, with each performed in triplicate. *P<0.05, **P<0.001

with smaller ones. This association of CI with higher serum PSA levels, larger prostate volumes, and bothersome LUTS requiring treatment by TURP in Taiwanese BPH patients supports the hypothesis that CI could lead to progression of BPH.

Although clinical evidence supports CI as a key factor leading to BPH, the underlying mechanism leading to differential prostate growth in widely distributed chronic inflammatory microenvironment is still unclear. Our comparative analysis of the gene expression profiles in BPH specimens from prostates of different size adds to a body of research that confirms the relationship between prostate size and activation of inflammatory genetic networks (34-36). Together, these studies provide genetic evidence that inflammation/immune pathways might be involved in

development and progression of BPH and PCa.

Consistent with genetic evidence, Kramer and colleagues demonstrated that BPH tissue contains infiltrates of T cells, B cells, and macrophages that are chronically activated and responsible for the release of cytokines (mostly IL-2, interferon-γ, and TGFβ1) that may support fibromuscular growth in BPH (37). Among these immune cells Th17 cells that provide defense against extracellular pathogens through release of IL-17 other cytokines (38). Notably, the levels of IL-17 are negligible in normal prostate but highly increased in BPH tissues (5). IL-17, which activates the NF-κB pathway, regulates the expression of IL-6, IL-8, and IL-1 in epithelial, endothelial, and stromal cells (13,16,39,40); thus, IL-17 is considered a key cytokine in BPH development and progression. These previous studies, together with our
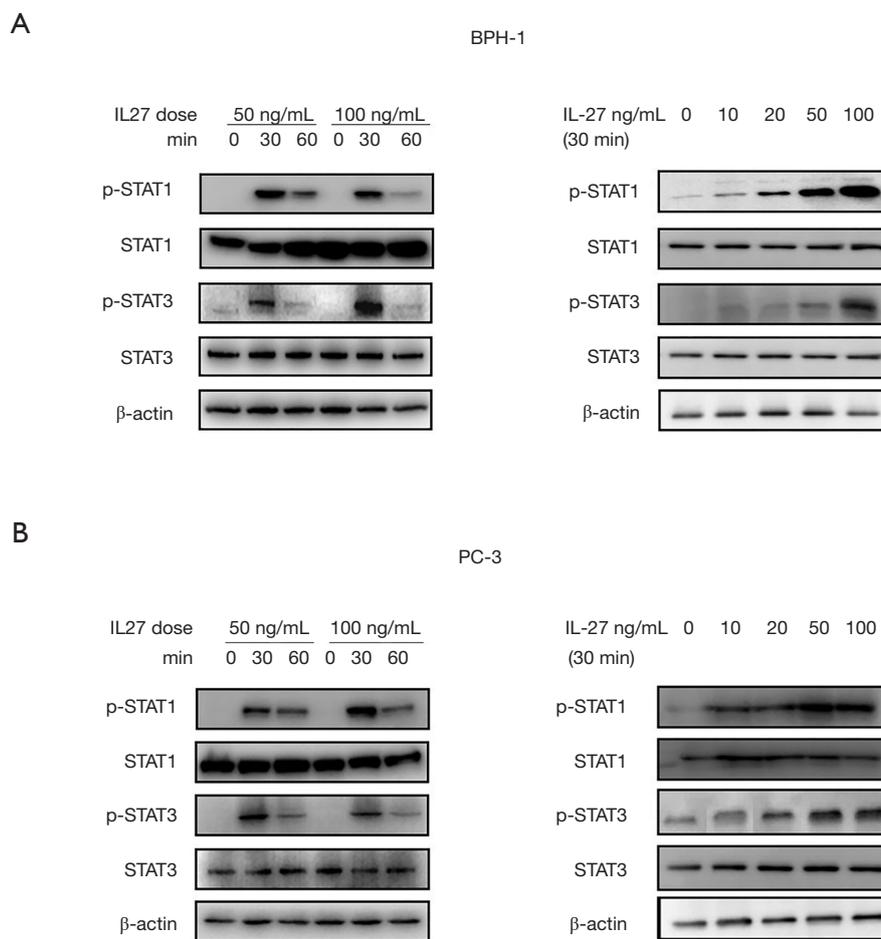
Figure 5 Phosphorylation of signal transducer and activator of transcription 1 (STAT1) and signal transducer and activator of transcription 3 (STAT3), the downstream effectors of IL-27/IL-27RA signaling, in BPH-1 and PC-3 cells after IL-27 stimulation. (A) The levels of phosphorylated STAT1 (p-STAT1) and phosphorylated STAT3 (p-STAT3) and total STAT1/STAT3 in BPH-1 cells were determined by western blotting at different time points (0, 30, and 60 min) and stimulated with different concentrations of IL-27 (0, 10, 20, 50, and 100 ng/mL). (B) The levels of p-STAT1/p-STAT3 and total STAT1/STAT3 in PC-3 cells were examined by western blotting with the same conditions as the BPH-1 cells. Data was representative of more than three independent experiments, with each performed in triplicate.

findings on the response to LPS via the TLR4 pathway, suggest a model in which prostate epithelial cells act as both sensors of infection and inducers of immune/inflammatory responses by expressing cytokines. These cytokines attract additional immune cells to augment inflammatory processes by expressing more potent cytokines, which stimulates epithelial cells in a vicious cycle (*Figure 6A*). These complex activities and interactions contribute to a chronic state of inflammation in BPH.

Interestingly, the expression of IL-17 signaling-associated genes was not significantly changed in our microarray data, suggesting that other cytokines contribute to the progression of BPH. We found that IL-27/IL-27RA signaling could modulate the production of LPS/TLR4 pathway-induced proinflammatory cytokines IL-6 and IL-8. IL-27, a pleiotropic cytokine that regulates multiple aspects of innate and adaptive immunity, has been associated with several inflammatory disorders including psoriasis (41), eczematous skin lesions (42), and periapical lesions (43). IL-27 is also part of the immune response to incoming pathogens. Studies have shown that IL-27 is induced by stimulation of TLR ligands or infectious agents (44-46). Although IL-27 is primarily produced by activated antigen presenting cells including dendritic cells and macrophages,
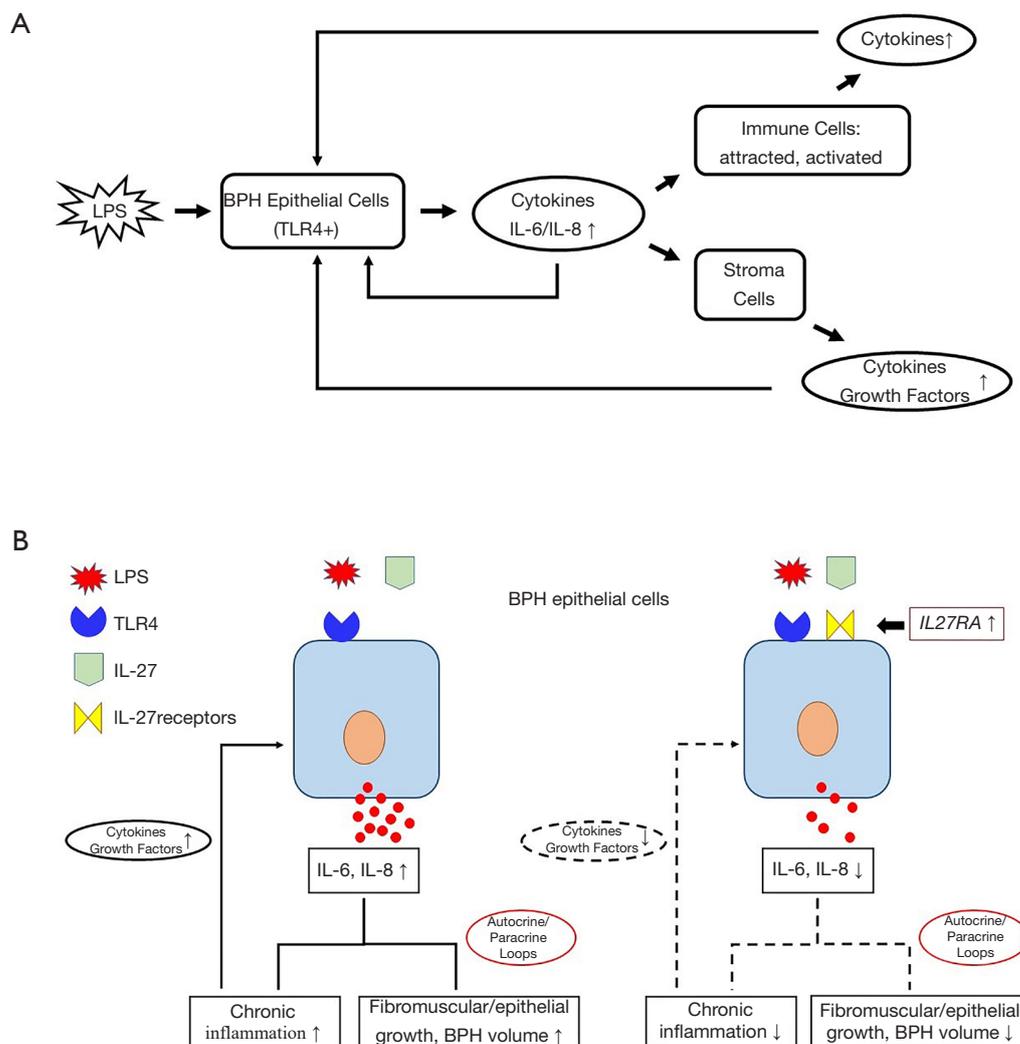
Transl Cancer Res 2020;9(8):4618-4634 | http://dx.doi.org/10.21037/tcr-20-1509

Figure 6 Schematic diagrams illustrating the complex activities and interactions contributing to a chronic state of inflammation in benign prostatic hyperplasia (BPH), and interleukin-27 (IL-27)/IL-27 receptor, alpha subunit (IL-27RA) signaling in modulation of inflammatory responses in BPH-1 cells and chronic inflammation in BPH progression. (A) Exogenous factors, such as LPS, activate the TLR4 pathway in BPH epithelial cells and induce production of inflammatory cytokines, which stimulate prostatic stromal and immune cells, and develop complex activities and feedback interactions that contribute to a chronic state of inflammation in BPH. (B) Left: In epithelial cells of BPH with less or absent expression of IL-27RA, Lipopolysaccharide (LPS)-activated toll-like receptor 4 (TLR4) signaling pathways induce production of proinflammatory and proliferative cytokines, interleukin-6 (IL-6) and IL-8, which lead to increased chronic inflammation and progression of BPH. Right: In epithelial cells of BPH with more or prominent expression of IL-27RA, activated IL-27/IL-27RA signaling suppresses production of IL-6 and IL-8 induced by the LPS/TLR4 pathway and attenuates chronic inflammation and progression of BPH.

several studies have revealed the capability of non-immune cells to express IL-27 (47,48). Additionally, the IL-27 receptor is found not only on innate or adaptive immune cells, but also on non-hematopoietic cells such as epithelial cells, fibroblasts, and keratinocytes (48). Furthermore, recent studies have shown that IL-27 has dual functions in proinflammatory and anti-inflammatory processes in adipocytes (49), oral epithelial cells (50), synovial cells (51), and intestinal epithelial cells (52). To our knowledge, the role of IL-27/IL-27RA signaling in CI of the prostate is previously unreported. Our results suggest a key role of IL-27/IL-27RA signaling in a negative feedback loop, which

could modulate infectious or inflammatory responses in prostate cells. Therefore, we speculate that inside BPH tissues, when the activated TLR4 pathway induced IL-6 and IL-8 production by prostate epithelial cells, the IL-27 was also expressed by antigen-presenting or epithelial cells. The differential expression of *IL-27RA* leads to different degrees of the activated IL-27/IL-27RA signaling, differentially suppressing the TLR4 pathway in prostate epithelial cells, and attenuating proinflammatory responses and immune activation in BPH tissues (*Figure 6B*) as well as in PCa.

In the present study, both in microarray and in cell biology experiments, our data revealed that IL-6 and IL-8 play major roles in the immune/inflammatory responses of prostate epithelial cells. IL-6 is a pleiotropic cytokine influencing antigen-specific immune responses and inflammatory reactions as well as hematopoiesis, bone metabolism, and neural development (53). At the beginning of acute inflammation (AI), IL-6 mediates the acute phase responses. When its activity as a proinflammatory cytokine persists, AI turns into CI that includes immune responses (54). The coexistence of IL-6 secretion and IL-6R expression on BPH stromal and epithelial cells suggest a probably paracrine and autocrine epithelial cell growth regulatory loop (55). Similarly, IL-6 is also secreted by neoplastic prostate epithelial cells and can stimulate PCa growth and progression through autocrine cross-activation of androgen receptor (56) and insulin-like growth factor-I receptor (IGF-IR) signaling (57). On the other hand, IL-8 is a proinflammatory cytokine, often induced along with IL-6 in response to chemical and microbial stimuli and selected cytokines. IL-8 stimulates stromal and epithelial growth by directly promoting the proliferation of senescent epithelial cells (58) and the stromal acquisition of a myofibroblast-reactive phenotype (59), and by indirectly inducing fibroblast growth factor-2 (FGF-2) secretion (60). The importance of IL-8 in prostatic CI was demonstrated by its increased levels in seminal plasma (61) and expressed prostatic secretion (62) in chronic prostatitis/chronic pelvic pain syndrome and in BPH. Thus, IL-8 is a reliable marker of BPH with CI, based on its positive correlation with IPSS and serum PSA. In addition, IL-8 is thought to be an important factor in tumor metastasis and resistance to treatment (63). The circulating level of IL-8 is increased in advanced PCa at the stage of hormone refractory and androgen independent metastasis (64). Thus, the expression of IL-8 may have significant prognostic value for PCa growth and response to therapy (65,66).

There are several limitations to this study. First, it is hard to ascertain the origins of infection/inflammation and sources of IL-27 in complex BPH tissues by only microarray studies, and thus we acknowledge that validation of the modulatory mechanism of IL-27/IL-27RA signaling would require more experiments and acquire more samples from a large population of BPH patients. Second, while the expression of IL-27RA is similar in RWPE-1 and BPH-1 cells (*Figure 2B*), the immunobiological features of IL-27 signaling in these two benign prostate epithelial cell lines might be indistinguishable. On the other hand, it is well known that prostatic inflammation is involved in the carcinogenesis and progression of PCa and might be a link between BPH and PCa (11,14,67). Thus, we chose to compare the effects of IL-27/IL-27RA signaling on LPS/TLR4 pathway-induced inflammatory responses in BPH-1 cells with PC-3 cells. Third, differential expression of *IL27RA* in the glandular epithelium of BPH might suggest the different activated status of IL-27 signaling between individuals facing infection versus CI. To further explore the role of IL-27RA, knockdown or overexpression of *IL27RA* in the components of BPH tissue would be required in future work.

In conclusion, our study is the first report that *IL27RA* is a biomarker of CI and a predictor of progression in BPH tissues. The gene expression of *IL27RA* is down-regulated in larger prostates and correlated with increased serum PSA and a higher prevalence of CI. In cell biology experiments, IL-27/IL-27RA signaling could modulate LPS/TLR4 pathway-induced proinflammatory responses by suppressing IL-6 and IL-8 production in prostate cells. These data enrich our knowledge of the role of CI in BPH, and highlight a novel function of activated IL-27/IL-27RA signaling in BPH tissues. These results suggest a novel mechanism of IL-27-mediated innate immunity during prostate infection/inflammation, which might be promising for the prevention and/or treatment of BPH.

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Table S1 Genetic networks of genes differentially expressed between large and small prostate glands

Network	Gene	Top functions	Score
1	ATP5F1, BXDC2, CNBP, CUL5, CUL4A, EIF1AX, EIF3A, EIF3M, EIF4E, Eif4g, EIF4G2, ERH, GDI2, HSPE1, LRRC41, MCTS1, MRPL14, NFkB (complex), NUDT21, PEBP1, RAB6A, RFWD2 (includes EG:64326), RPL3, RPL31, RPL37A (includes EG:6168), RPS16, RPS3A, SAR1A, SNIP1, TNFRSF14, TRIM56, UBE4A, VHL, WBP5, ZFAND6	Protein Synthesis, Gene Expression, RNA Trafficking	63
2	Akt, AKTIP, Alpha catenin, CAST, CHD1 (includes EG:1105), CLK1, DDX17, DEK, E2f, GNB2L1, GOLT1B, Hat, HAT1, Hdac, HDAC2, HISTONE, Histone h4, IFRD1, MORF4L1, N-cor, p70 S6k, PIK3R1, PRPF4, Rb, RBBP4, RBM7, SFRS3, SFRS12, SMARCA1, SMARCA2, TADA1L, TAF7, TAF9, TRA2B, ZRANB2	Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair	40
3	26s Proteasome, ADH5 (includes EG:128), Alcohol group acceptor phosphotransferase, ATPase, CCNC, CDK7, CDKN1B, DYRK1A, ERK, GTF2H1, Hsp70, IL27RA, MED1, MED16, MNAT1, PRKAA1, Proteasome, PSMC5, PSMC6, PSMD10, RANBP9, Rar, RNA polymerase II, RPN2, Sos, SP4, SPAST, TCEA1, TERF2IP, TFIIH, TSC1, TXNIP, UBA3, Ubiquitin, VitaminD3-VDR-RXR	Cell Signaling, Gene Expression, Cell Cycle	37
4	ACAA2, Ap1, APEX2, ARL6IP5, B2M, C12ORF23, CD164, CLIP1, FAM177A1, FAM35A (includes EG:54537), FSH, Histone h3, HMGB1 (includes EG:3146), IgG, IL12 (complex), Interferon alpha, KDM3A, KLRA17 (includes EG:170733), MAGED2, Mapk, MHC Class II, MIR124, MTMR6, NACA, Pdgf, PECI, PLOD3, PTBP1, RAVER2, RRN3, SEC31A, SET, SMAD5, SP3, ZBTB44	Cell Morphology, Cellular Assembly and Organization, Cardiac Inflammation	34
5	Actin, ACTR1A, ANK3, ATF4, ATXN1, Calmodulin, Calpain, Caspase, CK1, Ck2, CTNNAL1, CTNNB1, Cytochrome c, DCTN6, Fgf, HNRNPC, HTR2A, Jnk, KTN1, LDHB, LIN7C, MIR1, NARS, NLK, Pl3K, Pka, PLEKHA5, PP1-C, PPP1CC, RAB8B, RABL2A, RPLP2, SARS, TRIM2, XRCC5	Cellular Assembly and Organization, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function	33
6	CKS2, CPT2, CSDA, DAAM1, DDX17, DECR1, EMD, ERBB2, ESR1, G3BP1, LCOR, MAP4, MED25, MRC1, MXI1, MYBL1, MYBL2, MYC, NARS, NPTN, NUP210, PHF5A, PXN, RPL7, RPS24 (includes EG:6229), SFRS3, SPAG1, SPTLC1, SYCP3, TLN1, TMPO, TRIP12, WISP2, ZFR, ZNF622	Cancer, Reproductive System Disease, Tumor Morphology	22
7	ARMC1, ARNT, C4ORF34, CDC6, CDK5RAP3 (includes EG:80279), DUSP11, FAM103A1, GATA4, GPKOW, HDAC3, HIAT1 (includes EG:64645), HNF4A, HNRNPC, KPNB1, LSM4, LSM5, MIR146A, NBPF3, NMD3, NUP62, PHB (includes EG:5245), PLSCR1, PRCP, RHOXF2, SART3, SFRS1, SKP2, TNFSF13, TUBE1, UFM1, XPO1, ZC3H15, ZNHIT3, ZRANB2	RNA Post-Transcriptional Modification, Gene Expression, Cancer	21
8	ACOT7, ACOT1 (includes EG:641371), ARD1A, BLVRB, CNOT2, CNOT3, CNOT6, CNOT8, CNOT6L, CYP2B6, DDX27, DSN1, EGLN2, HNF4A, KBTBD7, LARP4, MIS12, MTF2, NAT13, NSL1, NUF2, NUP54, NUTF2, Palmitoyl-CoA hydrolase, PKP2, PMS1, POLR1B, POLR3F, POLRMT, SFRS11, TRDMT1, TXNDC9, UCHL1, USP5	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry	21
9	APH1A (includes EG:51107), ARHGAP12, CDH13, CSDA, FAM40A, FGFR1OP2, FXR2, HDHD2, HIF1A, ILK, KIAA1370, LRSAM1, MIR292, MIRN140, MTMR9, MYL1, NCSTN, NT5C2, P4HA1, PDCD10, PHF23, phosphatidylinositol-3,4,5-triphosphate, PPP1R14B, Procollagen-proline dioxygenase, PTPN13, RP5-1000E10.4, RPL13A, RPL5 (includes EG:6125), RTN3, SACM1L, SLC2A4, SNX1, TIMM8A, TRAF3IP3, VPS26A	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry	19
10	ATIC, ATP5J2, ATPase, BAT1, DDX19B, EPO, GNPNAT1, HABP2, HLTF, HNRPDL, IDE, IER3IP1, IFT74, KLHDC2, MIRN349, MUDENG, MYH1, MYH9, MYO9B, ONECUT1, OSBPL1A, PHB2, PPP2R4, PSMC1, PSMC5, PSMC6, RCC2, RPS4Y2, RWDD2B, SLC4A1, SPAST, TM2D1, TRAF6, VCP, WRB	Cellular Function and Maintenance, Hematological Disease, Cell Morphologyt	19

Table S2 Canonical pathways of genes differentially expressed between large and small prostate glands

| Canonical pathway	−log(P value)	Associated gene number
TREM1 Signaling	6.58+E00	13
LXR/RXR Activation	6.29+E00	19
Communication between Innate and Adaptive Immune Cells	6.16+E00	14
IL-10 Signaling	5.53+E00	13
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	5.49+E00	22
Acute Phase Response Signaling	5.48+E00	21
Hepatic Fibrosis/Hepatic Stellate Cell Activation	4.91+E00	18
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	4.79+E00	28
Interferon Signaling	4.45+E00	8
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	4.27+E00	12
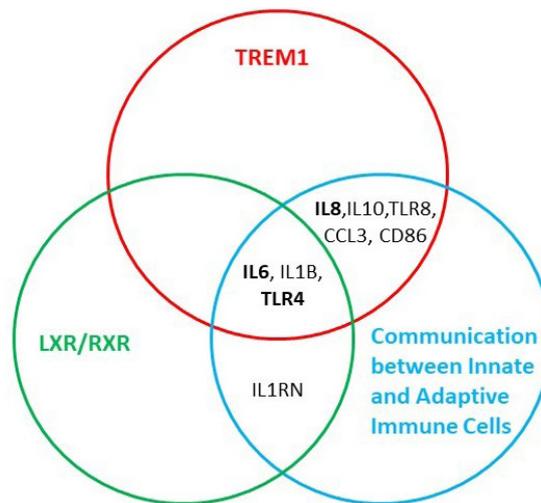
Figure S1 The genes among the top 3 canonical pathways whose expression simultaneously changed. Differentially expressed genes that were common among top 3 pathways were selected to design cellular experiments to validate the role of IL-27RA in mediating immune-related inflammation and effecting growth of prostate glands.