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Upregulation of TLR5 Indicates a Favorable Prognosis in Prostate Cancer

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Abstract

Background: Toll-like receptors (TLRs) are the key sensors of innate immunity for triggering immune responses against infections. TLRs are well known to be expressed and activated in innate immune cells, such as macrophage and dendritic cells, but we and others have found that some TLRs are also functional in epithelial cells (1–4). However, the role of an epithelial TLR in prostate cancer remains elusive (4).

Methods: TLR5 expression in mRNA and protein level in prostate cancer was determined by RT-qPCR and immunohistochemistry (IHC). The activation of TLR5 signaling in epithelial cells was detected upon NF-κB activation by luciferase assay and western blotting, and proinflammatory cytokine activation by RT-qPCR. Distinguishing between the TLR5 and NLRC4 pathways, both recognizing flagellin, is determined by siRNA and proinflammatory cytokine activation. The role of TLR5 in prostate cancer was analyzed by IHC and bioinformatics using a general and single-cell database.

Results: In the present study, we show that TLR5, amongst other TLRs, is exceedingly expressed in human prostate cancer cells. This cancer epithelial cell TLR5 functions to activate the TLR5 signaling pathway in human prostate cancer cells, as it does with innate immune cell TLR5. The bacterial protein flagellin induces a robust immune response in prostate cancer cells in a TLR5-dependent but NLRC4-independent manner. TLR5 is highly expressed in prostate cancer patient specimens, and high TLR5 expression in prostate cancer patients indicates a favorable prognosis.

Conclusions: TLR5, as an innate immunity receptor, is a functional TLR in human prostate cancer epithelial cells. TLR5 plays an important role in prostate cancer development and is a new potential prognosis biomarker. TLR5 may represent a novel immunotherapy target against prostate cancer.

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Declaration of competing interest

The authors declare that they have no potential conflict of interest in this paper.

Keywords

Toll-like receptor 5; prostate cancer; flagellin; prognosis; innate immunity; cancer immunity

1 Introduction

Cancer has long been one of the deadliest diseases in the world and the elusiveness of the tumors has made it an enigma to even modern medical treatments. With the great effort of scientists, the treatment and prevention of cancer have been improved dramatically. However, most cancers are not curable. Over half a million deaths every year in America alone are attributed to cancer, branding it the second leading cause of death marginally following heart disease (5). Shockingly, a woman in the U.S. has a thirty-nine percent chance of being diagnosed with some type of cancer in her lifetime, whereas a man has even more than a forty-five percent chance (6). Prostate cancer is one of the most common cancers in men. Out of one hundred men, about thirteen will develop prostate cancer during their lifetime, and among them, two to three will die of prostate cancer (7). Therefore, a novel strategy for cancer treatment is essential to improve the treatment and prevention of this disease.

Cancer immunotherapy is a novel therapy to utilize our own immune system to destroy tumor cells, and this new therapeutic strategy has shown promise for more effective treatment (8, 9). Current cancer immunotherapy which specifically targets adaptive immune T cells has given high hope to millions of cancer patients, but recent studies show that cancer immune therapy has not worked well in solid tumors including prostate cancer. Toll-like receptors are a family of transmembrane receptors and have been recognized as key sensors of innate immunity. The crucial role of Toll-like receptors (TLRs) in innate and adaptive immune responses has led to our hypothesis that some TLR agonists can be used in boosting anti-tumor immunity.

Recent studies have expanded the concept that inflammation is a critical component of tumor progression (10). For example, prostate cancer is typically associated with a chronic bacterial infection of the prostate characterized by recurrent urinary tract infections. In addition, tumor cells have co-opted some of the signaling molecules of the innate immune system, such as cytokines, chemokines, and their receptors for invasion, migration, and metastasis.

Innate immunity not only provides the first line of anti-microbial host defense but also has a profound impact on the induction of adaptive immune responses (11–13). Upon infection, microorganisms are first recognized by cells of the host’s innate immune system, such as macrophages, dendritic cells, as well as endothelial, and mucosal epithelial cells (14–16). Recognition of pathogens is primarily mediated by a set of germline-encoded molecules on innate immune cells that are referred to as pattern recognition receptors (PRRs) (17, 18). These PRRs are expressed as either membrane-bound or soluble proteins that recognize invariant molecular structures called pathogen-associated molecular patterns (PAMPs) (17, 18). Examples of PAMPs include lipopolysaccharides (LPS), lipoproteins (BLP), peptidoglycans (PGN), lipoteichoic acid (LTA), unmethylated CpG DNA, flagellin,

lipoarabinomannan (LAM) of mycobacteria, mannans of yeast, profilin of parasite, viral double-stranded RNA, and viral single-stranded RNA (19, 20). It is already clear that TLRs have a crucial role in the recognition of 'molecular signatures' of infecting microbes, in engaging differential signaling pathways, and in controlling the maturation of dendritic cells and the differentiation of T helper cells (21, 22). Elucidation of the molecular mechanisms enhancing host immunity against tumors provides possible immunotherapeutic approaches against cancer (23, 24).

We and others have previously found some TLRs were not only expressed in innate immune cells but also in cancer epithelial cells (2, 25). We, among the first groups, found that the epithelial TLR5 can be activated to inhibit breast cancer cell proliferation and tumor growth (2). However, it is not clear if a TLR can be a therapeutic target for prostate cancer. In a mouse model, normal prostate tissue shows a strong expression of TLR5 (26). When the prostate tumor progressed in a mouse model, the expression level of TLR5 decreased in prostate tissues and tumor tissues (26). Thus, it is critical to investigate the TLR expression profile on human prostate tumor tissues and the potency of a TLR agonist to inhibit human prostate tumor development. This study aims to determine whether a TLR and its activated signaling pathway play an important role in prostate epithelial cells and prostate cancer development. Hence, providing a new potential therapeutic target against prostate cancer.

2 Material and Methods

2.1 The study approvals

All the experiments in this study have been approved by our institute research committee with documentary number IBC2018-075 (expiration date: 11/03/2024).

2.2 Reagents and Cell lines

TLR5 ligand *Salmonella typhimurium* flagellin was purchased from InvivoGen (San Diego, CA, USA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Human prostate cancer cell line LNCaP (ATCC CRL-1740) and DU-145 (ATCC HTM-81) were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, USA). The cells were maintained in a humidified incubator at 37°C in a 5% CO_2 incubator. The cells from frozen stocks were generated within two to three passages and were negative for mycoplasma. Cell lines were not externally authenticated.

2.3 RNA isolation and reverse transcription

Total RNA from human prostate cancer LNCaP and DU-145 cells was isolated with E.Z.N.A. Total RNA Kit from Omega Bio-tek (Norcross, USA), following its corresponding protocol. cDNA was prepared by Oligo$(dT)_{12–18}$ and reverse transcriptase SuperScript II from Invitrogen with 2 μg of DNase I-treated total RNA.

2.4 Semi-quantitative RT-PCR

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the expression of TLRs in different human prostate cancer cells (Fig. 1B). The primers are as seen in "Table 1". PCR parameters were as follows: denaturation at 94°C for 3 min was followed by 25 or 35 cycles at 94°C for 20 s, at 57°C for 20 s, and at 72°C for 30 s. The PCR products were subjected to electrophoresis in 1.5% agarose gels, visualized under UV light after ethidium bromide staining, and then imaged. All assays were performed at least three times from independent RNA preparations. The PCR products had been verified in our previous reports (2, 27, 28).

2.5 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was used to determine TLR5 signaling activation by checking downstream cytokine TNF-α expression. RT-qPCR was performed on a Bio-Rad iQ5 Real-time PCR system with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Amplification was achieved using an initial cycle of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Gene expression was normalized using β-actin as an internal reference gene control. All assays were performed at least three times from independent RNA preparations. Expression was calculated according to the relative $\Delta\Delta C_t$ method as described previously (2). The RT-qPCR primers used for the analysis of TNF-α expression are listed in Table 1.

2.6 NF-κB Luciferase Assays

The luciferase assay was conducted as described previously (29), with NF-κB luciferase reporter construct pBIIX (A gift from Dr. Sankar Ghosh who was at Yale University and is at Columbia University currently) (1). Briefly, the human prostate cancer cells (from ATCC) were transiently transfected with 0.05 μg of NF-κB luciferase reporter construct pBIIX and 0.2 μg of a construct directing expression of *Renilla* luciferase under the control of the constitutively active thymidine kinase promoter (pRL-TK; Promega) and plated into 24-well tissue culture plates. Tumor cells were starved with 1% FBS medium for 2 hours to decrease the signaling background and then treated with flagellin at different concentrations as seen in Figure 1 for 6 hours. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Data are presented as the mean ± S.D. of triplicate samples and are representative of three independent experiments.

2.7 Immunohistochemistry

Human prostate cancer Formalin-fixed paraffin-embedded tissue arrays were purchased from US Biomax. Immunohistochemical analyses were performed with the ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) as previously reported (2). Slides were first deparaffinized, then Heat-induced antigen retrieval was performed using 0.1 M citrate buffer (pH 6.0) and lastly autoclaved for 20 minutes. Endogenous peroxidase was eliminated with 3% H_2O_2. Then, washed with the phosphate-buffered saline (PBS) and blocked with normal goat serum (1%) plus 1% bovine serum albumin (BSA) in PBS for 20 minutes at room temperature. Slides were then incubated overnight with primary antibodies (1:200) against

TLR5 in 1x PBS with 1% BSA. Samples were washed with PBS and incubated with biotin anti-rabbit secondary antibody (1:500) for 1 hour. Next, samples were washed with PBS and incubated with streptavidin–horseradish peroxidase conjugate solution for 20 minutes. After washing with PBS, the slides were then mounted with a mounting solution. The TLR5 expression level in all the sections was evaluated by two independent investigators in a double-blind manner and staining intensity was graded as negative and positive (including weak and strong).

2.8 MTT assay

DU-145 cells were seeded into a 96-well plate at 2,500 cells per well. The cells were treated with PBS or flagellin (0.1 μg/ml) 24 hours before the MTT assay. To measure cell proliferation, the cells were incubated with an MTT reagent (AMRESCO, Solon, OH, USA) for 3 hours on days 0, 2, 4, and 6. The absorbance was measured at 570nm on the indicated days. Each assay was performed in triplicates.

2.9 ELISA

After the DU-145 cells were stimulated with the same volume of either flagellin (0.1 μg/ml) or PBS for 24 hours, levels of the secreted TNF-α and IL-6 proteins in the supernatants were determined by the human TNF-α and IL-6 ELISA kits from BD Biosciences.

2.10 TCGA data and Single-cell RNA sequencing analysis

We collected the data from a total of 494 prostate cancer patients in The Cancer Genome Atlas (TCGA) database and analyze the expression of TLR5 in these patients (Data are made available in supplementary Table 1). Single-cell RNA-seq data were downloaded from the GEO databases [GSE172357 (30), GSE176031 (31)] and normalized by SCT transformation. For single cell analysis, there are 6 samples from organ donors and 13 samples from benign prostatic hyperplasia patients in GSE172357. And there are 20 samples from prostate cancer patients (patients 1–7) in GSE176031 (Data are made available in supplementary Table 2). Data analysis was performed on the Grace portal of Texas A&M's high-throughput research computing system, using R and RStudio with the Seurat package (version 4.0.6) (32). The batch effect was corrected with the Harmony package (33). The cell types were manually identified and epithelial cells were identified with classical markers, including EPCAM, KRT5, KRT8, and KRT18.

2.11 Western Blotting

The expression of IκBα and phosphate p65 proteins in DU-145 and LNCaP cells were detected by western blot analysis. Briefly, the cell lysate proteins (40 μg) were used for each sample. Proteins were resolved in 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 1% BSA in TBST buffer for 1 hour at room temperature. Primary antibody (from Cell signaling) diluted 1:1000 in 1% BSA was added and incubated at 4°C overnight. The membranes were washed 3 times with TBST for 5 min. The HRP-secondary antibody was then added and incubated at room temperature for 1 hour, followed by detection with ECL. The image was taken by FuoChem M System (Bucher Biotec).

2.12 siRNA knockdown

Cells were transfected with scramble siRNA, TLR5 siRNA, and NLRC4 siRNA from Horizon (a PerkinElmer company), according to the manufacturer's instructions. The efficiency of the knockdown was verified by RT-qPCR.

2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0. (GraphPad Software, La Jolla, CA USA). Data are represented as mean ± s.e.m. and p-values for differences in expression were determined by the data analyzed using a two-tailed unpaired Student's *t*-test or Mann–Whitney *U* test. Kaplan-Meier survival curves with long-rank tests were used to compare survival differences between the high-expression and low-expression groups.

3 Results

3.1 The expression and activation of TLRs in prostate cancer epithelial cells

TLRs are key microbial sensors in the innate immune system. TLRs are mainly expressed and activated in antigen-presenting innate immune cells such as macrophages and dendritic cells. Surprisingly, we and others found that TLRs are also expressed in epithelial cells (1–4). It has been well known that innate immunity including TLRs plays an important role in prostate cancer (4). But it has not been well determined whether a TLR is functional in prostate cancer cells. Here, we pursued the expression and functional status of TLRs in human prostate cancer cells in order to identify a potential novel immunotherapeutic target. First, we transferred an NF-κB luciferase reporter into prostate cancer DU-145 and LNCaP cells. Among TLRs, only TLR5 can be activated inducing NF-κB activation in prostate cancer cells when they are stimulated by their agonists (Figure 1A). Then, we utilized the traditional semi-quantitative RT-PCR to quickly check the expression status of all human TLRs (TLR1 to TLR10) in human prostate cancer cell lines, including LNCaP and DU-145. All the PCR reactions used the same master mix except for the individual TLR primers, thus different TLRs themselves might serve as a perfect internal control of each other. Strikingly, we found that among the TLRs, TLR5 was highly expressed in human prostate cancer DU-145 and LNCaP cells, appearing even in 25 cycles of PCR reaction (Figure 1B). At this time point of PCR, we could not detect the expression of other TLRs. Our further analyses identified some other TLRs including TLR1, TLR3, TLR6, and TLR9 that were also expressed in LNCaP or DU-145 cells in 35 cycles of PCR reaction (Figure 1B). The broad expression of TLRs in human prostate tumor cell lines suggests the role of TLRs signaling in prostate cancer progression, in which the exceedingly expressed TLR5 might play an important role in human prostate cancer development and progression.

3.2 Epithelial cell TLR5 was a functional TLR in human prostate cancer cells

After uncovering that TLR5 was exceedingly expressed in comparison to other TLRs, next we wanted to further verify that TLR5 in human prostate cancer cells is functional, like with macrophages and dendritic cells. TLRs in innate immune cells demonstrate the capacity to recognize TLR ligands and activate TLR signaling. The activated TLR signaling generally induces the activation of the master transcriptional factor NF-κB and further

induces proinflammatory cytokines, such as TNF-α, IL-6, IL-12, etc. We investigated whether TLR5 activation in human prostate cancer cells occurred by stimulating the cells with the TLR5 ligand flagellin, a bacterial protein, in a dosage-dependent manner. DU-145 cells were treated with flagellin in different dosages, and we checked the NF-κB activation by luciferase assay (Fig 2A) and NF-κB activation by Western blotting for IκBα and phosphate p65 (Fig. 2B).

The results showed that after treatment with flagellin, a strong NF-κB luciferase activation occurred in a dose-dependent manner (Fig 2A) and induced NF-κB activation at the protein level (Fig. 2B) in both DU-145 and LNCaP cells. NF-κB activation was not detected in PC3 cells (Fig 2A). The reason why some prostate cancer cells like PC3 do not respond to flagellin is unclear. We then checked the downstream proinflammatory cytokines including TNF-α and IL-6 activation by RT-qPCR and ELISA (Fig 3A–C). We found that flagellin induced TNF-α and IL-6 expression significantly in prostate cancer cells both at the mRNA level and the protein level (Fig 3A–C). To further verify this finding, we found that flagellin induced TNF-α in a dose-dependent manner (Fig 3A). These results further confirmed that TLR5 was a functional TLR in human prostate cancer cells. Furthermore, flagellin treatment does not affect cell proliferation in DU-145 cells (Fig 3D), indicating no overt toxicity of flagellin to the cells. We then wanted to see whether the expression and functional status of TLR5 in prostate cancer cells can reflect the real situation in human prostate cancer patients. Flagellin can activate both the TLR5 signaling pathway and the NLRC4 pathway. We then checked which pathway was activated in flagellin-induced proinflammatory cytokines in prostate cancer cells. By using siRNA knockdown of TLR5 or NLRC4 specifically, we identified that flagellin activated the TLR5 signaling pathway but not the NLR4 pathway (Figure 4). Therefore, we next checked the TLR5 expression in human prostate cancer specimens.

3.3 TLR5 was upregulated in prostate cancer epithelial cells and prostate cancer specimens

The high expression and activation of TLR5 in prostate cancer cell lines led us to investigate whether this status was reflected in patients with prostate cancer. Public single-cell RNA sequencing data were analyzed to further determine the expression of TLR5 in the prostate cancer epithelial cells (Supplemental Table 1). No noticeable difference in TLR5 mRNA expression was observed between healthy prostate and benign prostatic hyperplasia (Fig. 5A). However, TLR5 was upregulated in prostate cancer epithelial cells (Fig. 5B).

Furthermore, we performed immunohistochemical staining with TLR5-specific antibodies on tissue arrays from US Biomax to investigate TLR5 expression in human prostate specimens. The tissue arrays included some normal human prostate tissues, but they were not paired with the prostate cancer specimens seen in Table 2. Thus, we could not compare the expression level of TLR5 in normal and tumor tissues. Overall, we found that normal prostate tissues expressed a low level of TLR5, while prostate tumor tissue expressed an elevated level of TLR5 (Fig 5C). We cannot conclude that the TLR5 expression in tumors is higher than in normal controls because the tissue specimens are not paired. However, our findings might indicate that up-regulation of TLR5 plays a role in human prostate cancer

development, although more specimens in different stages are needed to draw a definite conclusion (Table 2). We were eager to see whether the upregulated TLR5 in prostate cancer was beneficial or harmful to patient prognosis, so we next investigated this using publicly available databases.

3.4 Upregulated TLR5 in prostate cancer indicated a favorable prognosis

We found that TLR5 was highly expressed in 80% of prostate cancer specimens, but our tissue sample size was small. To further confirm our findings, we searched the publicly available TCGA database. First, we analyzed all human TLRs (from TLR1 to TLR10) in the human prostate cancer database, including a total of 494 human prostate cancer specimens with TLR5 expression information (380 high, 76.9%; 114 low, 23.1) (Supplemental Table 2). After analysis of these data, TLR5 played a significant role in prostate cancer (p = 0.037) (Fig 6A). Then, the data showed that human prostate cancer patients with high TLR5 expression had a much better prognosis (Fig 6B). Thus, upon stimulation, TLR5 on prostate cancer cells can successfully activate TLR5 signaling which could aid in prostate cancer patient prognosis and TLR5 might represent a new potential therapeutic target against prostate cancer.

4 Discussion

Innate immunity is a new potential therapeutic target against cancer. Here, we first explored whether TLRs are expressed in prostate cancer epithelial cells. Surprisingly, we identified that several TLRs are expressed in different prostate cancer cells. In particular, TLR5 was found to be higher expressed in prostate cancer cells such as LNCaP and DU-145 as well as the prostate tumor specimens from patients. More importantly, we found that the TLR5 ligand flagellin, a bacterial protein, has the capacity for activating TLR-specific signaling in human prostate cancer cells DU-145 and LNCaP, which is consistent with the previous report (34). In that study, they are the first to report that TLR3 and TLR5 are functional TLRs in prostate cancer cells (34). Furthermore, they found that after the activation of TLR3 and TLR5 in the prostate cancer cells, the cells release chemokines to recruit specific immune cells (34). In our study, we confirmed the role of TLR5 in prostate cancer cells and showed that flagellin induced proinflammatory cytokines in a TLR5-dependent and NLRC4-independent manner. We further investigated TLR5's role on prostate cancer patients with tissue specimens and TCGA data by performing single-cell analysis and functional analysis. Thus, collectively these studies together strongly indicate that TLR5 can be a good prognostic biomarker and a novel potential therapeutic target for prostate cancer. We also showed that activation of TLR5 in DU-145 cells increased cytokine TNF-alpha expression significantly. Autocrine secretion of cytokines by cancer cells exerts an important effect in the control of cancer cell behavior such as adhesion and invasion (35, 36). As prostate cancers are aggravated under conditions of chronic urinary infection (37), our study suggests that TLR5 activation might have a crucial role in prostate cancer development and provide a direct link between innate immune response and tumor progression (Fig 6C). It is to be noted that the human TLR5 and mouse TLR5 might demonstrate different functions. We reported that TLR11 belongs to the TLR5 subfamily in mice but not in humans and has a similar function to human TLR5 (1). TLR11 in humans is a pseudogene (1). Furthermore,

we cannot make a conclusive statement that the TLR5 expression level in prostate cancer is higher than in normal prostate because we cannot collect enough pairs of human samples, and the tissue array samples are not paired. The public data set is not paired too. But based on the data available, prostate cancer patients with high expression of TLR5 survived better than those with low expression of TLR5 (Fig 5B.). Thus, we conclude that TLR5 might have an important role in controlling the development of human prostate cancers.

It is striking to identify the difference between non-tumorigenic and tumorigenic cells in terms of TLR5 expression. The TLR5 signaling in prostate cancer can be utilized to stimulate innate immune responses, inhibiting prostate cancer development. Of course, the bright line between stimulating a safe immune response to curtail the effects of cancer and putting the body in an auto-immune state must also be identified. Our work paves the foundations for improved prostate cancer vaccine development. Provenge is the first therapeutic cancer vaccine for patients with advanced prostate cancer to receive approval from the U.S. FDA. However, the vaccine prompts only a partial response, favoring B cells over T cells. TLR5 manipulation enables kick-starting the inflammatory response and stimulates the adaptive immune system to pump memory cells. Compounds that activate TLR5 specifically might enable the body to develop a stronger immune response against tumorigenic cells. The stimulation and challenge to both the innate and the adaptive immune systems to recognize cancer cells is a small step in a marathon to find the cure, but once this step is taken, the marathon toward successful immunotherapy is started.

Among the common treatments for prostate cancer patients, chemotherapy uses medication to kill tumor cells or slow the growth of a tumor, and radiotherapy uses high-energy rays to destroy or impede tumor growth, whereas surgery cuts out the primary tumor. However, the increasing mortality and morbidity rates indicate that a novel and more effective alternative treatment for cancer is eagerly desired. Yet that brings up the question: what if white blood cells can be taught to recognize cancer cells faster and respond at a quickened pace? The crucial role of Toll-like receptors (TLRs) in innate and adaptive immune responses has led to our hypothesis that TLR agonists can be used in boosting anti-tumor immunity. In our studies, we have found that several TLRs are expressed in prostate cancer cells, and specifically, TLR5 activation leads to a robust immune response. Therefore, modulation of TLR5 activation on human prostate cancer might have the potential to improve the efficacy of prostate cancer vaccines. Efforts to harness the immune system for cancer therapy have tremendous potential, although immunotherapy faces daunting challenges.

The present study faces some limitations. The discovery of the role of epithelial TLR5 in prostate cancer development is intriguing, but our tissue array specimens do not include paired normal prostate samples. We cannot definitely conclude that the TLR5 expression in prostate cancer is higher than in normal prostate. Although we analyze the TCGA and single-cell RNA sequencing data to find that TLR5 activation in prostate cancer indicates a favorable prognosis, the exact role and mechanisms of epithelial TLR5 will have to be verified *in vivo*. This *in vivo* study is ongoing, but the microenvironment between human prostate cancer and mouse prostate cancer model is remarkably different. A recent report has shown that bacteria-associated cancers are much higher than we thought before and that bacterial footprints like LPS can be detected in about 60–70% of many kinds of

cancers (38). Additionally, the function of TLR5 is quite different between humans and mice. Therefore, the phenotype in the animal model might be different (39, 40). We will consider these issues and test different models as we investigate the role of TLR5 *in vivo*. Furthermore, our ongoing research aims to determine how TLR5 is activated in prostate cancer development, in which we will try to identify the endogenous ligand of TLR5 in prostate cancer development.

5 Conclusions

TLR5, as an innate immunity receptor, is a functional TLR in human prostate cancer epithelial cells. TLR5 plays an important role in prostate cancer development. TLR5 is a new potential prognosis marker for human prostate cancer and might represent a new potential therapeutic target against prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TLR	Toll-like receptor
PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
TCGA	the Cancer Genome Atlas

References

1. Zhang D, Zhang G, Hayden MS, et al. A toll-like receptor that prevents infection by uropathogenic bacteria. Science. 2004;303(5663):1522–1526. [PubMed: 15001781]
2. Cai Z, Sanchez A, Shi Z, Zhang T, Liu M, Zhang D. Activation of Toll-like receptor 5 on breast cancer cells by flagellin suppresses cell proliferation and tumor growth. Cancer Res. 2011;71(7):2466–2475. [PubMed: 21427357]
3. Shi Z, Cai Z, Yu J, et al. Toll-like receptor 11 (TLR11) prevents Salmonella penetration into the murine Peyer patches. J Biol Chem. 2012;287(52):43417–43423. [PubMed: 23135279]
4. Zhao S, Zhang Y, Zhang Q, Wang F, Zhang D. Toll-like receptors and prostate cancer. Front Immunol. 2014;5:352. [PubMed: 25101092]
5. CDC. An Update on Cancer Deaths in the United States. In. https://www.cdc.gov/cancer/dcpc/research/update-on-cancer-deaths/2022.
6. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65(1):5–29. [PubMed: 25559415]

7. Force USPST, Grossman DC, Curry SJ, et al. Screening for Prostate Cancer: US Preventive Services Task Force Recommendation Statement. JAMA. 2018;319(18):1901–1913. [PubMed: 29801017]
8. Filin IY, Solovyeva VV, Kitaeva KV, Rutland CS, Rizvanov AA. Current Trends in Cancer Immunotherapy. Biomedicines. 2020;8(12).
9. Igarashi Y, Sasada T. Cancer Vaccines: Toward the Next Breakthrough in Cancer Immunotherapy. J Immunol Res. 2020;2020:5825401. [PubMed: 33282961]
10. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860–867. [PubMed: 12490959]
11. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003;3(2):133–146. [PubMed: 12563297]
12. Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta. 2002;1589(1):1–13. [PubMed: 11909637]
13. Shi M, Zhang Y, Liu L, et al. MAP1S Protein Regulates the Phagocytosis of Bacteria and Toll-like Receptor (TLR) Signaling. J Biol Chem. 2016;291(3):1243–1250. [PubMed: 26565030]
14. Beutler B. Toll-like receptors: how they work and what they do. Curr Opin Hematol. 2002;9(1):2–10. [PubMed: 11753071]
15. Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol. 2002;14(1):103–110. [PubMed: 11790539]
16. Vasselon T, Detmers PA. Toll receptors: a central element in innate immune responses. Infect Immun. 2002;70(3):1033–1041. [PubMed: 11854180]
17. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54 Pt 1:1–13.
18. Medzhitov R, Janeway CA Jr. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol. 1997;9(1):4–9. [PubMed: 9039775]
19. Janeway CA Jr., Medzhitov R. Lipoproteins take their toll on the host. Curr Biol. 1999;9(23):R879–882. [PubMed: 10607553]
20. Anderson KV. Toll signaling pathways in the innate immune response. Curr Opin Immunol. 2000;12(1):13–19. [PubMed: 10679407]
21. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1(2):135–145. [PubMed: 11905821]
22. Tohno M, Shimosato T, Moue M, et al. Toll-like receptor 2 and 9 are expressed and functional in gut-associated lymphoid tissues of presuckling newborn swine. Vet Res. 2006;37(6):791–812. [PubMed: 16973119]
23. Hajam IA, Dar PA, Shahnawaz I, Jaume JC, Lee JH. Bacterial flagellin-a potent immunomodulatory agent. Exp Mol Med. 2017;49(9):e373. [PubMed: 28860663]
24. Cui B, Liu X, Fang Y, Zhou P, Zhang Y, Wang Y. Flagellin as a vaccine adjuvant. Expert Rev Vaccines. 2018;17(4):335–349. [PubMed: 29580106]
25. Mokhtari Y, Pourbagheri-Sigaroodi A, Zafari P, Bagheri N, Ghaffari SH, Bashash D. Toll-like receptors (TLRs): An old family of immune receptors with a new face in cancer pathogenesis. J Cell Mol Med. 2021;25(2):639–651. [PubMed: 33336901]
26. Nguyen CT, Hong SH, Sin JI, et al. Flagellin enhances tumor-specific CD8(+) T cell immune responses through TLR5 stimulation in a therapeutic cancer vaccine model. Vaccine. 2013;31(37):3879–3887. [PubMed: 23831323]
27. Xia B, Shan M, Wang J, et al. Homeobox A11 hypermethylation indicates unfavorable prognosis in breast cancer. Oncotarget. 2017;8(6):9794–9805. [PubMed: 28038461]
28. Huang X, Shi Z, Wang W, et al. Identification and characterization of a novel protein ISOC2 that interacts with p16INK4a. Biochem Biophys Res Commun. 2007;361(2):287–293. [PubMed: 17658461]
29. Cai Z, Shi Z, Sanchez A, et al. Transcriptional regulation of Tlr11 gene expression in epithelial cells. J Biol Chem. 2009;284(48):33088–33096. [PubMed: 19801549]
30. Joseph DB, Henry GH, Malewska A, et al. Single-cell analysis of mouse and human prostate reveals novel fibroblasts with specialized distribution and microenvironment interactions. J Pathol. 2021;255(2):141–154. [PubMed: 34173975]

31. Song H, Weinstein HNW, Allegakoen P, et al. Single-cell analysis of human primary prostate cancer reveals the heterogeneity of tumor-associated epithelial cell states. Nat Commun. 2022;13(1):141. [PubMed: 35013146]
32. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. Cell. 2021;184(13):3573–3587 e3529. [PubMed: 34062119]
33. Korsunsky I, Millard N, Fan J, et al. Fast, sensitive and accurate integration of single-cell data with Harmony. Nat Methods. 2019;16(12):1289–1296. [PubMed: 31740819]
34. Galli R, Starace D, Busa R, et al. TLR stimulation of prostate tumor cells induces chemokine-mediated recruitment of specific immune cell types. J Immunol. 2010;184(12):6658–6669. [PubMed: 20483744]
35. Sansone P, Storci G, Tavolari S, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest. 2007;117(12):3988–4002. [PubMed: 18060036]
36. Schafer ZT, Brugge JS. IL-6 involvement in epithelial cancers. J Clin Invest. 2007;117(12):3660–3663. [PubMed: 18060028]
37. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell. 2006;124(4):823–835. [PubMed: 16497591]
38. Nejman D, Livyatan I, Fuks G, et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. Science. 2020;368(6494):973–980. [PubMed: 32467386]
39. Han JH, Park JH, Kim BY, et al. Decreased expression of Toll-like receptor 4 and 5 during progression of prostate transformation in transgenic adenocarcinoma of mouse prostate mice. J Vet Sci. 2015;16(3):281–287. [PubMed: 25797291]
40. Zhao Y, Yang J, Shi J, et al. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature. 2011;477(7366):596–600. [PubMed: 21918512]

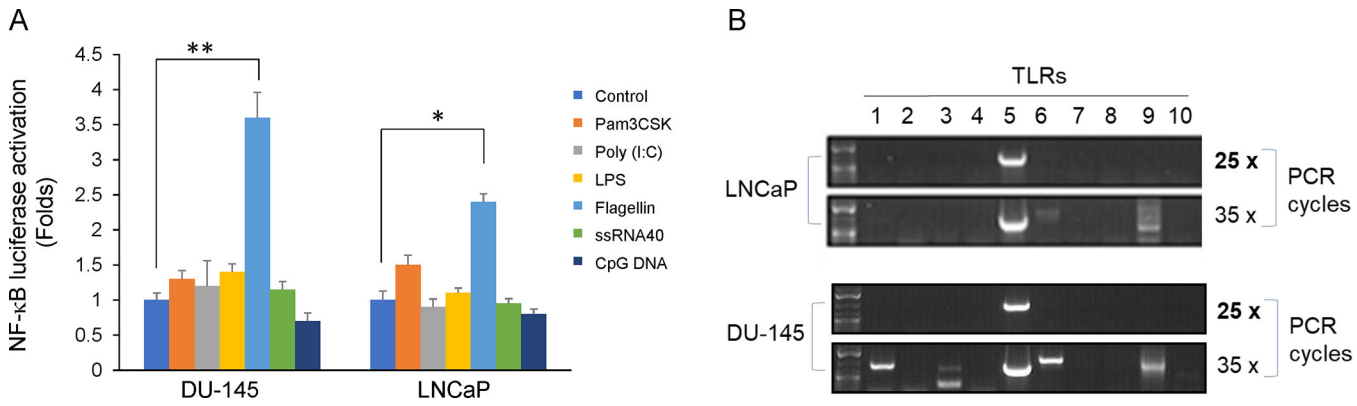

Figure 1. The expression and activation of TLRs in prostate cancer epithelial cells.

(A) NF-κB luciferase reporter was transfected into prostate cancer cells for 24 hours. The cells were then treated with different TLR ligands for six hours, including Pam3CSK (10 ng/ml), Poly (I:C) (100 ng/ml), LPS (100 ng/ml), Flagellin (100 ng/ml), ssRNA40 (1 μg/ml), and CpG DNA (ODN2006, 2 μM). NF-κB activity was determined by luciferase assay. (B) The expression profile of toll-like receptors (TLRs) in human prostate cancer LNCaP and DU-145 cells was determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Both LNCaP and DU-145 prostate cells predominantly expressed TLR5, as shown in the bands in 25 cycles of PCR product. All the PCR reactions used the same master mix except for the individual TLR primers, thus different TLRs from each other might serve as a perfect internal control. *p<0.05, **p<0.01, Student paired t test.

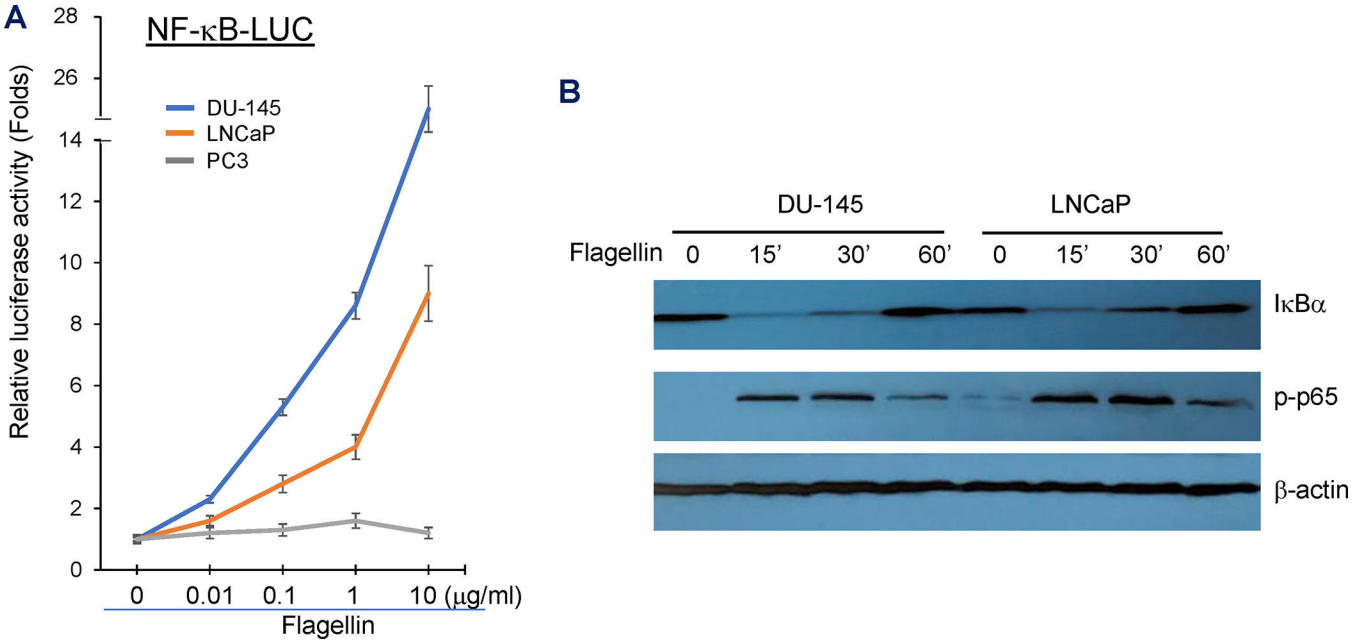

Figure 2. Flagellin induced NF-κB activation in human prostate cancer cells.

(A) NF-κB luciferase reporter was transfected into prostate cancer cells for 24 hours. The cells were then treated with different dosages of flagellin for six hours, and then NF-κB activity was determined by luciferase assay. (B) Western blotting was performed to determine NF-κB activation in prostate cancer cells.

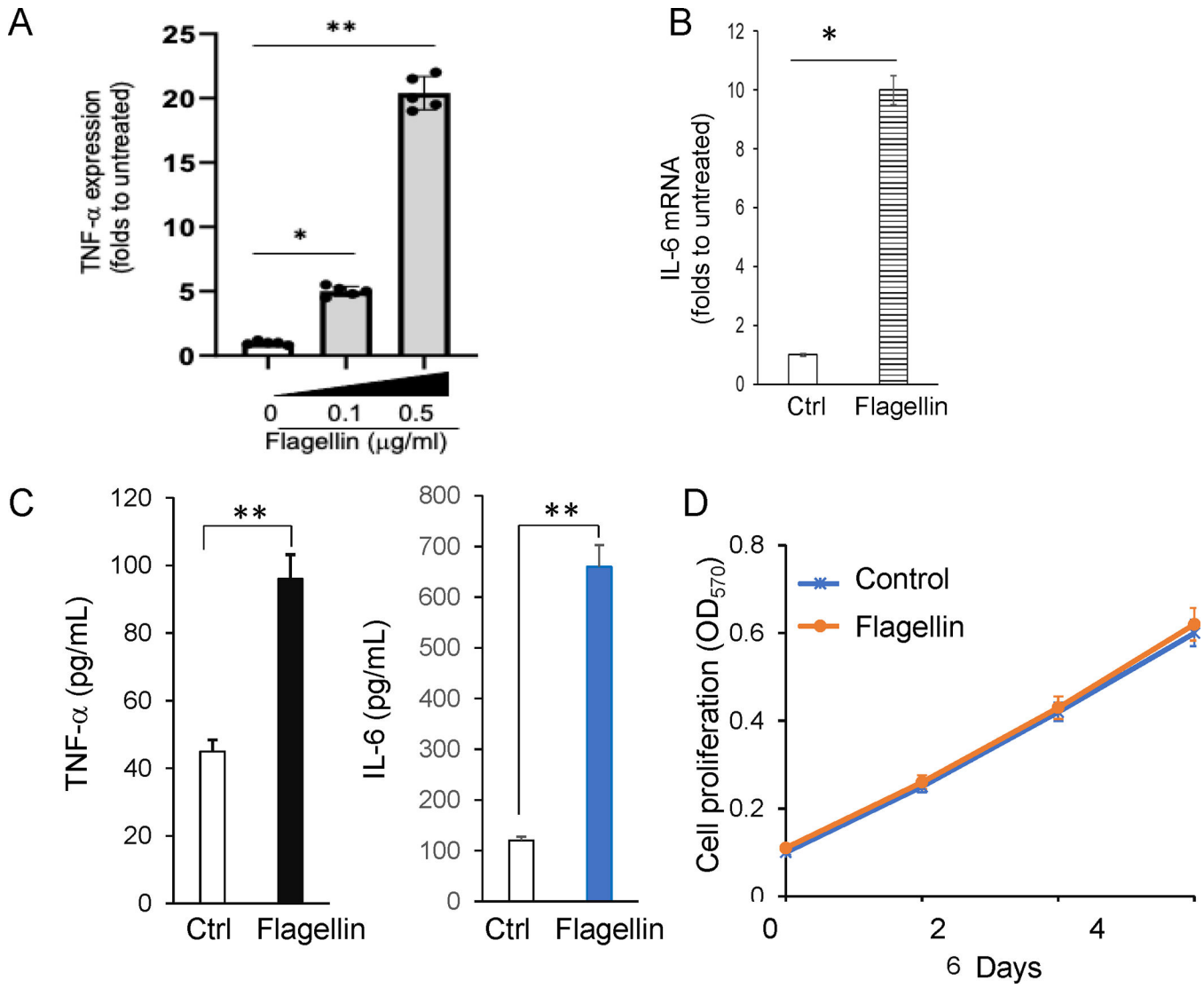

Figure 3. Flagellin activated the TLR5 signaling pathway inducing proinflammatory cytokines in human prostate cancer cells.

The activation of TLR5 signaling to induce proinflammatory cytokines was investigated in flagellin-activated human prostate cancer DU-145 cells. (A,B) DU-145 prostate cancer cells were treated with 0, 0.1, or 0.5 μg/ml flagellin and cultured for 2 hours. DU-145 cells were then harvested, and total RNA was isolated. TNF-α and IL-6 expression induced by flagellin in different dosages (A) or 0.5 μg/ml (B) in DU-145 prostate cells were determined by real-time quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR). *P<0.05, untreated group vs 0.1 μg/ml flagellin treated group. ** P<0.01, untreated group or 0.1 μg/ml flagellin treated group vs 0.5 μg/ml flagellin treated group. (C) TNF-α and IL-6 secretion in DU-145 cells treated with 0.1 μg/ml flagellin for 24 hours were determined by an enzyme-linked Immunosorbent assay (ELISA). Error bars show SEM. **P <0.01. (D) Flagellin treatment does not affect cell proliferation in DU-145 cells, which was determined by MTT assay.

Each dot in (A) represents a biological repeat. Data were analyzed by 2-tailed unpaired *t*-tests (A-C).

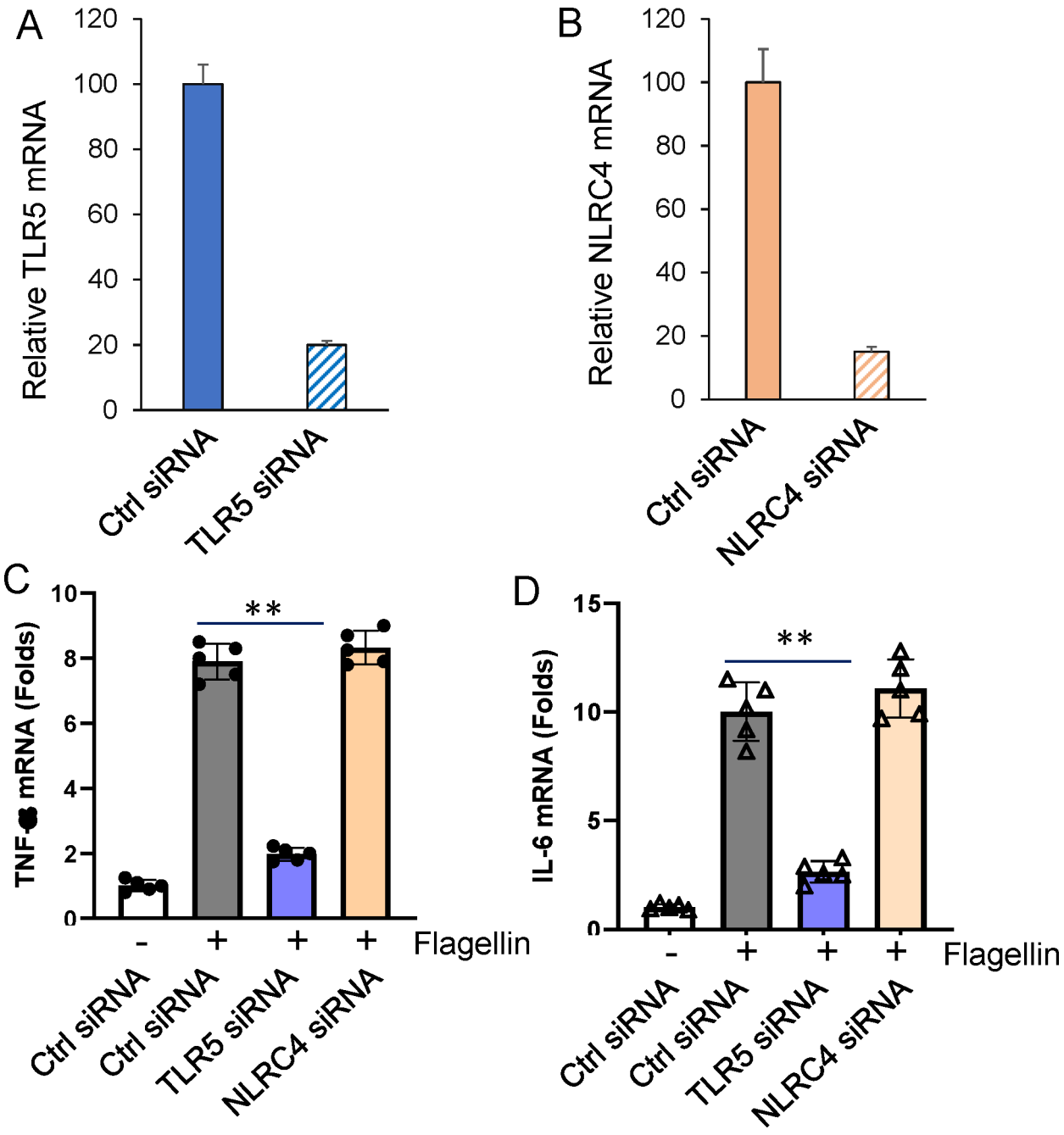

Figure 4. Flagellin induces proinflammatory cytokines in human prostate cancer cells in a TLR5-dependant and NLRC4-independent pathway.

(A,B) DU-145 prostate cancer cells were transfected with control siRNA, TLR5 siRNA, or NLRC4 siRNA, and were treated with 0.1 μg/ml flagellin and cultured for 2 hours. (C,D) TNF-α and IL-6 expression induced by flagellin in DU-145 prostate cells were determined by RT-qPCR. ** P<0.01, control siRNA group or TLR5 siRNA group.

Each dot in (C,D) represents a biological repeat. Data were analyzed by 2-tailed unpaired t-tests (C,D).

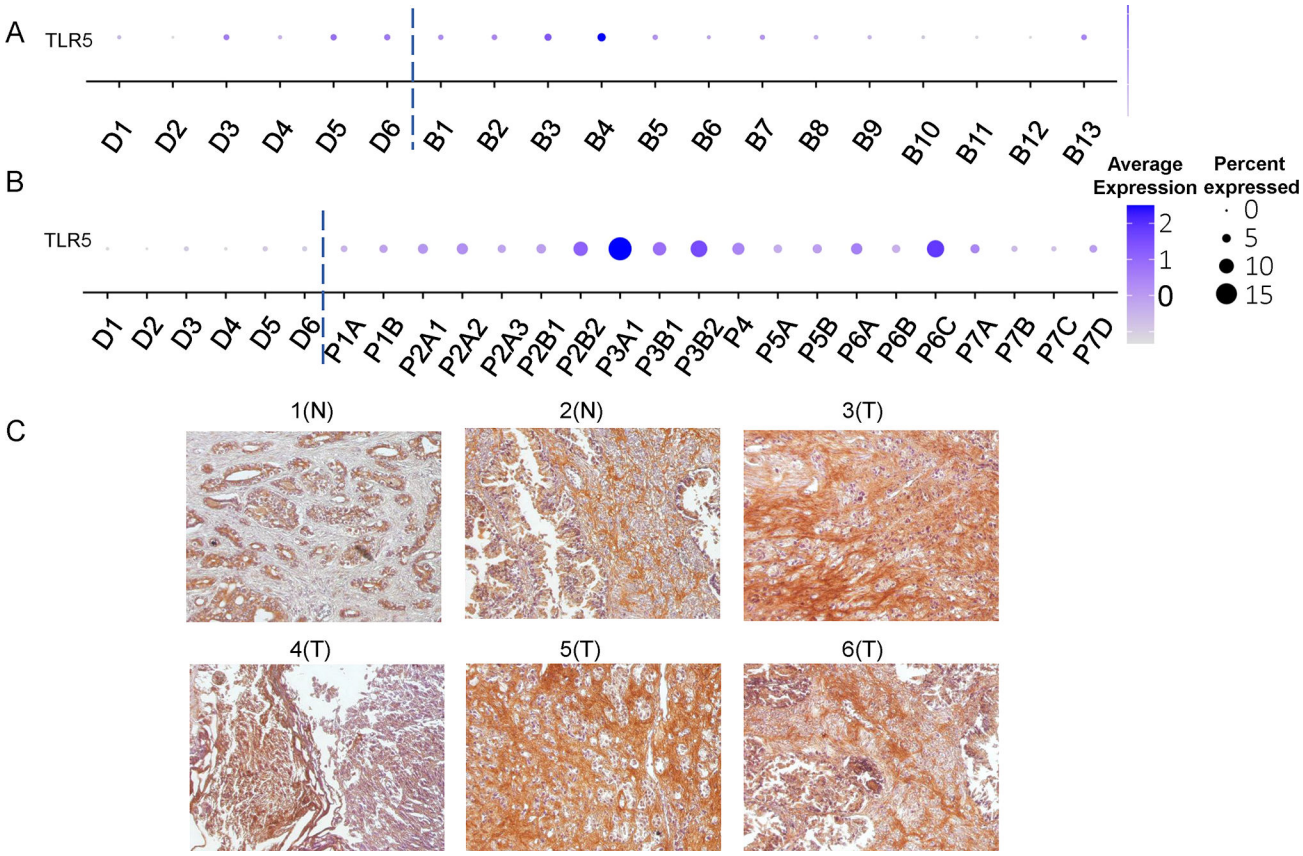

Figure 5. TLR5 activation in human prostate cancer patients.

(A,B) TLR5 was upregulated in prostate cancer epithelial cells by a single-cell RNA sequencing analysis. (A) The dot plot shows the expression of TLR5 in epithelial cells from normal prostate or benign prostatic hyperplasia. The single-cell RNA-seq data were collected from the GEO database (GSE172357). D: healthy donor; B: benign prostatic hyperplasia patient. (B) The dot plot shows the expression of TLR5 in epithelial cells from normal prostate or prostate cancer. The single-cell RNA-seq data were collected from the GEO database (GSE172357, GSE176031). D: healthy donor; P: Prostate cancer patient. (C) Human prostate cancer tissue arrays were subjected to immunohistochemistry analyses with a monoclonal primary antibody against TLR5 and a biotinylated second antibody. Representative images from normal prostate tissues (N) and unpaired prostate tumors (T) were shown. Brown indicates positive staining for TLR5. Representative images from tissues with different histological types are shown. Magnification, X40. The summary data of all the specimens were listed in Table 2.

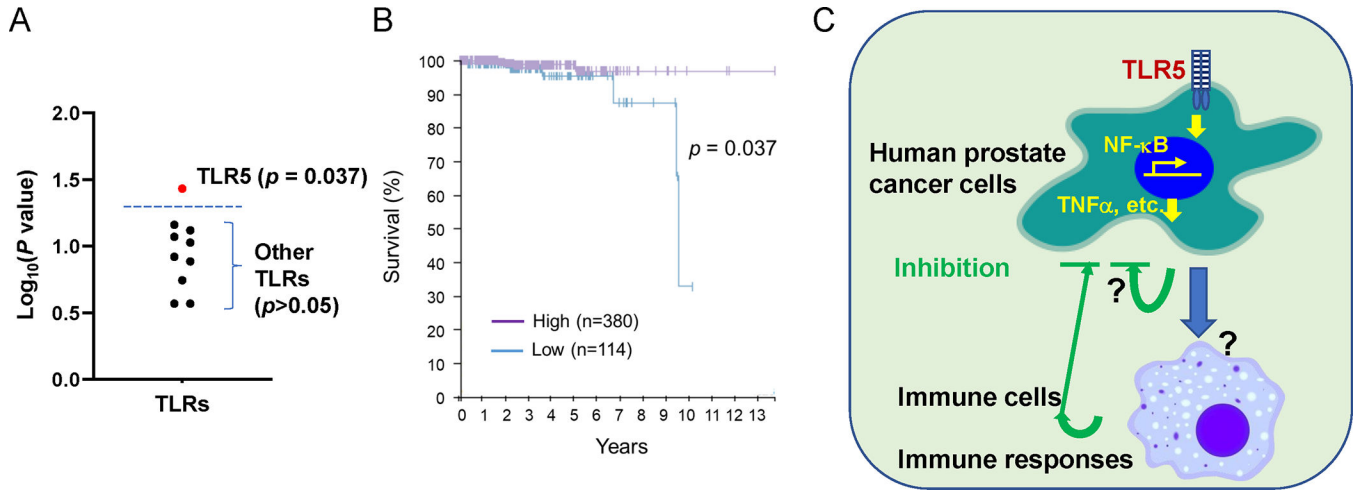

Figure 6. Upregulated TLR5 in prostate cancer indicated a favorable prognosis.

(A) By Analysis of different TLR roles in prostate cancer with TCGA data, only TLR5 played a significant role in prostate cancer (log-rank test: p=0.037). (B) The patients with high TLR5 expression had a much better prognosis. Kaplan-Meier survival curves with long-rank tests were used to compare survival differences between the high-expression (76.9%) and the low-expression group (23.1%). (C) A proposed working model for the role of epithelial TLR5 in prostate cancer.

Table 1.

Sequences of PCR Primers

Gene	Forward primer	Reverse primer
TLR1	CGTAAAACTGGAAGCTTTGCAAGA	CCTTGGGCCATTCCAAATAAGTCC
TLR2	GGCCAGCAAATTACCTGTGTG	CCAGGTAGGTCTTGGTGTTCA
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC
TLR4	CTGCAATGGATCAAGGACCA	TCCCACTCCAGGTAAGTGTT
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA
TLR6	TAGGTCTCATGACGAAGGAT	GGCCACTGCAAATAACTCCG
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG
TLR9	TTATGGACTTCCTGCTGGAGGTGC	CTGCGTTTTGTCGAAGACCA
TLR10	CAATCTAGAGAAGGAAGATGGTTC	GCCCTTATAAACTTGTGAAGGTGT
TNFα	CGAGTGACAAGCCTGTAGC	GGTGTGGGTGAGGAGCACAT
IL-6	GGAGACTTGCCTGGTGAA	GCATTTGTGGTTGGGTCA
NLRC4	TCTACCTGATCCAGCATTAGTCAG	TGCCACCCAACAAGCCTAGC
β-actin	TCCCTGGAGAAGAGCTACG	GTAGTTTCGTGGATGCCACA

Table 2.

Clinicopathologic features of TLR5 in patients with prostate cancer

	TLR5	
	Positive	**Negative**
	n= 32 (80%)	**n= 8 (20%)**
Age		
≤ 65	8 (20.0%)	5 (12.5%)
> 65	24 (60.0%)	3 (7.5%)
Tumor grade		
I	10 (25.0%)	3 (7.5%)
II	10 (25.0%)	5 (12.5%)
III	12 (30.0%)	0