Lymphotoxin β receptor activation promotes mRNA expression of RelA and pro-inflammatory cytokines TNFα and IL-1β in bladder cancer cells

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Received April 2, 2016; Accepted March 8, 2017

DOI: 10.3892/mmr.2017.6676

Abstract. The role of inflammation in tumorigenesis and development is currently well established. Lymphotoxin β receptor (LTβR) activation induces canonical and noncanonical nuclear factor (NF)-κB signaling pathways, which are linked to inflammation-induced carcinogenesis. In the present study, 5,637 bladder cancer cells were cultured and the activation of LTβR was induced by functional ligand, lymphotoxin (LT) α1β2, and silencing with shRNA. Reverse transcription-quantitative polymerase chain reaction was utilized to detect the mRNA expression levels of NF-κB family members RelA and RelB, cytokines including LTα, LTβ, tumor necrosis factor (TNF)α, TNF superfamily member 14, interleukin (IL)-6 and IL-1β, and proliferation-related genes including CyclinD1 and Survivin. The expression of phospho-p65 was determined by western blotting. Activation of LTβR on bladder cancer 5,637 cells was demonstrated to upregulate the mRNA expression levels of the RELA proto-oncogene, RelA, by 2.5-fold compared with unstimulated cells, while no significant change was observed in the RELB proto-oncogene NF-κB member mRNA levels. Expression of pro-inflammatory cytokines tumor necrosis factor (TNF)α and interleukin (IL)-1β mRNA levels were significantly increased nearly 5-fold and 1.5-fold, respectively, following LTβR activation compared with unstimulated cells. The LTβR-induced upregulation of RelA, TNFα and IL-1β was decreased by ~33, 27, and 26% respectively when LTβR was silenced via short hairpin RNA. Activation of LTβR had no effect on 5,637 cell growth, despite CyclinD1 and Survivin mRNA levels increasing by ~2.7 and 1.3-fold, respectively, compared with unstimulated cells. In conclusion, activation of LTβR induced the expression of RelA mRNA levels. LTβR activation might be an important mediator in promoting an inflammatory microenvironment in bladder cancer, via the upregulation of TNFα and IL-1β mRNA levels. LTβR may be a potential therapeutic target for bladder cancer.

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Key words: bladder cancer cell, lymphotoxin β receptor, nuclear factor-κB, cytokine, proliferation

Introduction

The role of inflammation in tumorigenesis and development is now well established in the biomedical literature. There is mounting evidence that an inflammatory microenvironment is an essential component of most tumor types, including some in which the causal relationship with inflammation remains to be elucidated (1). In some cancer types, inflammatory conditions precede development of malignancy; in others, oncogenic change induces a tumor-promoting inflammatory milieu (1,2). Globally, there are large variations in bladder cancer mortality (3,4), suggesting an important role of environmental factors in the etiology of bladder cancer. The common known risk factors of bladder cancer include a number of occupations with exposures to aromatic amines (for example, industrial dye manufacturing), the drug cyclophosphamide, cigarette smoking, and chronic infection (for example, urinary tract infections and schistosomiasis) (5). The unifying principle that underlies these risk factors is chronic inflammation, which is an aberrantly prolonged form of a protective response to the loss of tissue homeostasis (6). A previous study from our group reported great numbers of immune cells infiltrated in bladder cancer tissue from patients (7), suggesting a strong relationship between bladder cancer and chronic inflammation. Immune cells that infiltrate tumors engage in a dynamic crosstalk with cancer cells, resulting in the inflammatory tumor microenvironment. Understanding the underlying mechanisms of how immune cells function in different tumor types, and which molecular inflammatory mediators might drive or potentially prevent carcinogenesis, is a subject of intense research.

Lymphotoxin β receptor (LTβR) was initially discovered in the context of lymph node development, and it has since been demonstrated that LTβR signaling participates in the initiation and/or development of inflammation-induced carcinogenesis (8). LTβR is expressed in a wide range of tumor types, including breast, colorectal, lung, stomach, melanoma and bladder cancer (9,10), while its ligands, lymphotoxin (LT) a1b2 and TNF superfamily member 14 (TNFSF14; also known as LIGHT), are mainly expressed on the surface of immune cells (11). Thus, LTβR signaling might enable the communication between infiltrating immune cells and tumor cells (8). Triggering LTβR induces the canonical and noncanonical nuclear factor (NF)-κB signaling pathways, which are linked to inflammation-induced carcinogenesis (12). Sustained LTβR signaling leads to NF-κB-mediated chronic inflammation and hepatocellular carcinoma (HCC) development (13). Long-term suppression of LTβR with a LTβR agonistic antibody significantly reduces chronic hepatitis incidence and prevents the transition from chronic hepatitis to HCC in mouse models (13). By contrast, LTβR functions as a death receptor that mediates tumor cell apoptosis in colon carcinoma, mammary carcinoma and sarcoma (14); Yang *et al* (15) and Winter *et al* (16) reported that LTβR induces cytotoxic T lymphocyte-mediated antitumor cytotoxicity. Because of these contrasting observations, the function of LTβR signaling might be tumor type and cellular context-dependent. In addition, the function of LTβR remains unclear in bladder cancer. Therefore, the present study aimed to investigate the effect of LTβR activation on the mRNA expression levels of the NF-κB main members, RELA proto-oncogene (RelA; also known as p65) and RELB proto-oncogene (RelB) and to analyze the function of LTβR in the proliferation and the pro-inflammatory response in bladder cancer cells.

Materials and methods

Cell culture. Human bladder cancer 5,637 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in RMPI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) at 37˚C humidified atmosphere with 5% CO_2. For the present study, 5,637 cells were seeded onto 96-well plates, 24-well plates and 6-well plates at densities of 4-5x10^4 cells/ml, 2-5x10^5 cells/ml and 1-2x10^6 cells/ml respectively. Cells were then divided into two experimental groups, LTβR-activated and LTβR-silenced. Activation of LTβR was induced by addition of the functional ligand, lymphotoxin (LT) α1β2 (R&D Systems, Inc., Minneapolis, MN, USA), at a final concentration of 100 ng/ml. Silencing of LTβR was performed by specific LTβR small hairpin RNA (shRNA). Prior to each experiment, cells were grown in RPMI-1640 without FBS for 6 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 5,637 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Total RNA of each sample (2 μg) was subjected to oligo-dT-primed RT using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The resulting cDNA was diluted 1:20 used for qPCR, using a SYBR-Green Real-Time PCR Master mix on a 7500 Real-Time PCR System (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sequences were as follows: human LTβR, sense 5'-GCACAAGCAAACGGAAGACC-3' and antisense 5'-GAC CTTGGTTCTCACACCTGGT-3'; LTα, sense 5'-CTGCTC ACCTCATTGGAGAC-3' and antisense 5'-CCTGGGAGT AGACGAAGTAGAT-3'; LTβ, sense 5'-CAGCTGCCCACC TCATAGG-3' and antisense 5-GCGTCCGAGAACTGC GTC-3'; LIGHT, sense 5'-ATCTCACAGGGGCCAACT-3' and antisense 5'-ACGGACGACCACCTTCTC-3'; tumor necrosis factor (TNF)α, sense 5'-GCCCATGTTGTAGCA AACC-3' and antisense 5'-GGTAGGAGACGGCGATG-3'; IL-6, sense 5'-AATTCGGTACATCCTCGACGGC-3' and antisense 5'-GCCAGTGCCTCTTTGCTGCTTT-3'; interleukin (IL)-1β, sense 5'-GAAATGATGGCTTATTACAGT GGCA-3' and antisense 5'-GTAGTGGTGGTCGGAGATTCG TAG-3'; CyclinD1, sense 5'-TGCATCTACACCGACAAC TCC-3' and antisense 5'-GGGCGGATTGGAAATGAACT-3'; and Survivin, sense 5'-GACCACCGCATCTCTACATTCA-3' and antisense 5'-AATTCACCAAGGGTTAATTCTTCAA-3'. qPCR was performed under the following conditions: initial denaturation at 95˚C for 5 min, then 45 cycles of 95˚C for 30 sec and 60˚C for 30 sec. Melting curves were recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized to the level of GAPDH (sense 5'-GTC AACGGATTTGGTCGTATTG-3' and antisense 5'-CTGGAA GATGGTGATGGGATT-3'). Relative fold changes in mRNA expression were calculated using the formula $2^{-\Delta\Delta Cq}$ (17).

LTβR gene silencing by shRNA. The shRNA sequences were designed to exhibit sequence homology to the two LTβR transcripts (NM_0,01270987.1, NM_002342.2) at http://rnaidesigner.invitrogen.com/rnaiexpress/rnaiDesign.jsp (BLOCK-iT™ RNAi Designer; Thermo Fisher Scientific, Inc.). Bladder cancer 5,637 cells were transfected with LTβR-specific shRNA (5'-GCACCTATGTCTCAGCTA AAT-3', loop sequence is CGAA) plasmid as the tested group (shRNA-T), in parallel with scramble shRNA (5'-CTACAC AAATCAGCGATTT-3', loop sequence is CGAA) plasmid as the control group (shRNA-C). The day prior to transfection, 5,637 cells were seeded into 24-well (7-9x10^5 cells/ml) or 6-well (1-2x10^6 cells/ml) culture plates in complete media. The next day, shRNA (at final concentration 0.8 and 4 μM for 24-well and 6-well culture plates, respectively) were introduced into cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. At 4 h post-transfection, media were replaced with regular complete culture media. The cells were cultured for 72 h prior to analysis of the gene-silencing effects. The expression of LTβR in the shRNA-transfected cells was characterized by RT-qPCR and western blotting.

Western blotting. For determining the silencing effect of the shRNA plasmid, 5,637 cells were cultured in 6-well plates (1-2x10^6 cells/ml) and transfected with the shRNA for 72 h. Total cells were lysed on ice with radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Nantong, China) supplemented with a protease

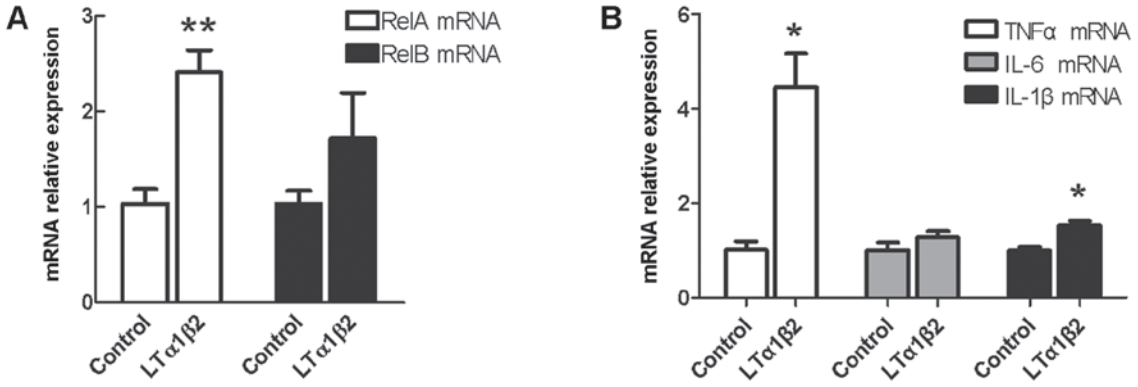

Figure 1. Effect of LTβR activation on mRNA expression of RelA/p65, RelA, and pro-inflammatory cytokines TNFα, IL-6 and IL-1β. Bladder cancer 5,637 cells were stimulated with 100 ng/ml LTα1β2 and mRNA expression levels were examined for (A) RelA and RelB, (B) TNFα, IL-6 and IL-1β mRNA. *P<0.05 and **P<0.01, compared with control unstimulated cells. Error bars represented the standard deviation. LTβR, lymphotoxin β receptor; RelA, RELA proto-oncogene NF-κB subunit; RelB, RELB proto-oncogene NF-κB subunit; TNFα, tumor necrosis factor αl; IL, interleukin; LT, lymphotoxin.

inhibitor cocktail (cat. no. I3786; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 10-15 min. The concentration of protein in the lysates was measured by an Enhanced Bicinchoninic Assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology). A total of 30 μg lysate was loaded onto each lane of 5% stacking gel and 8% separating gel and separated by SDS-polyacrylamide gel electrophoresis. Following SDS-PAGE, proteins were transferred onto polyvinylidene fluoride membranes (Solarbio Science and Technology Co., Ltd., Beijing, China). Membranes were subsequently blocked with 5% bovine serum album (BSA) (Generay Biotech Co., Ltd., Shanghai, China) diluted in TBST (10 mM Tris-HCL, 100 mM NaCl, 0.2% Tween-20) at 37˚C for 2 h. After blocking, membranes were incubated with primary antibodies against LTβR (cat. no. 20331-1-AP; ProteinTech Group, Inc., Chicago, IL, USA) diluted 1:500 in TBST containing 5% BSA and β-actin (cat. no. GTX124213; GeneTex, Inc., Irvine, CA, USA) diluted 1:5,000 in TBST containing 5% BSA overnight at 4˚C. Membranes were subsequently probed with a horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. A0208; Beyotime Institute of Biotechnology) diluted 1:1,000 in TBST containing 5% BSA at room temperature for 2 h. Immunoreactivity was visualized using an Enhanced Chemiluminescence reagent (cat. no. K-12045-C20; Advansta, Inc., CA, USA). Densitometric analysis was performed using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc., CA, USA).

Cell viability assay. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. Briefly, 5,637 cells were seeded in 96-well plates at a density of 4-5x10^4 cells/ml and stimulated with LTα1β2 for 24 and 48 h. Control cells were treated with equal volumes of sterile PBS. At the indicated time point, the culture media was aspirated and fresh media supplemented with 10% (v/v) CCK-8 was added to the cells. Cells were cultured at 37˚C for an additional 3 h and the absorbance was then measured at a wavelength of 450 nm. Cell viability was expressed as % of the control unstimulated culture measurement.

Statistical analysis. All statistical analyses were performed using SPSS version 15.0 software (SPSS, Inc., Chicago, IL, USA). Independent t tests between two groups were performed to determine statistical significance. P<0.05 was considered to indicate a statistically significant difference. Experiments were repeated three times.

Results

Activation of LTβR enhances the mRNA expression of RelA, but not of RelB. Activation of NF-κB signaling occurs by two pathways, the canonical pathway and the noncanonical signaling pathway. In order to examine the effect of LTβR activation on the expression of the main members of the canonical and noncanonical NF-κB signaling pathways, the RelA and RelB genes were selected as target genes, respectively. As demonstrated in Fig. 1A, RelA mRNA expression levels were upregulated by 2.5-fold following activation of LTβR in bladder cancer 5,637 cells, compared with control unstimulated cells (P=0.003). No significant difference was observed in the expression of RelB mRNA following LTβR activation compared with control unstimulated cells (P=0.254; Fig. 1A).

Activation of LTβR promotes the mRNA expression of pro-inflammatory cytokines TNFα and IL-1β. It is known that cytokines are major mediators of communication between cancer cells and immune cells in the inflammatory tumor microenvironment (2). The hypothesis that major pro-inflammatory cytokines, TNFα, IL-6 and IL-1β, which are also target genes of NF-κB signaling, might be regulated by LTβR was examined. As demonstrated in Fig. 1B, mRNA expression levels of TNFα were increased by ~5-fold (P=0.034) and IL-1β by 1.5-fold (P=0.013) following LTβR activation compared with control unstimulated cells. No effect on the expression of IL-6 mRNA was observed (P=0.334; Fig. 1B).

LTβR-induced upregulation of RelA, TNFα and IL-1β is reversed by LTβR silencing. To determine whether activation of LTβR has a causative role in the upregulation of NF-κB signaling members and pro-inflammatory cytokines, LTβR was silenced in 5,637 cells by shRNA. LTβR-specific shRNA (shRNA-T group) and non-silencing shRNA plasmids (shRNA-C group) were transfected into the 5,637 cells. The efficiency of LTβR silencing was confirmed at the mRNA

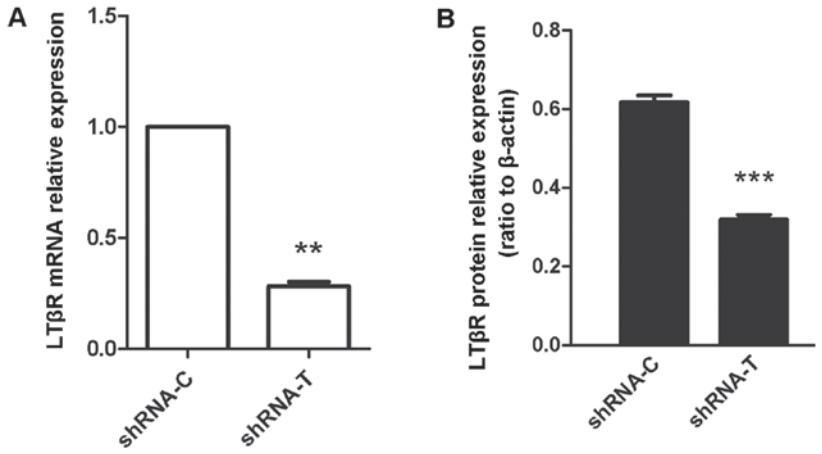

Figure 2. Efficiency of LTβR silencing via shRNA. Bladder cancer 5,637 cells were transfected with LTβR-specific shRNA plasmid (shRNA-T group) or with control scramble shRNA plasmid (shRNA-C group) for 72 h, then (A) mRNA and (B) protein expression of LTβR was examined. **P<0.01 and ***P<0.001, compared with shRNA-C group. Error bars represented the standard deviation. LTβR, lymphotoxin β receptor; shRNA, short hairpin RNA.

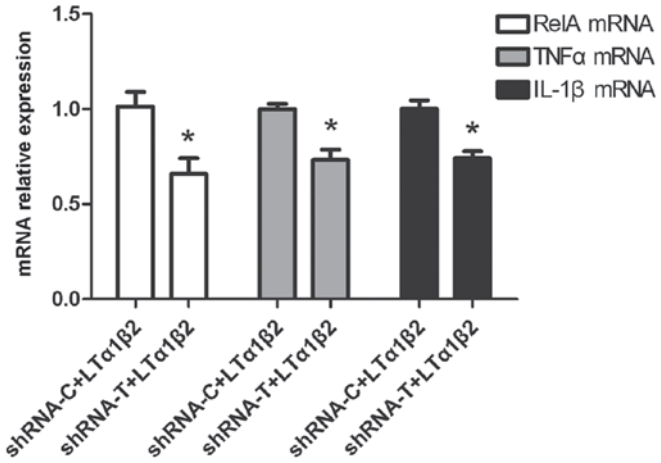

Figure 3. Effect of LTβR silencing on the LTα1β2-mediated overexpression of RelA, TNFα and IL-1β. Following LTβR silencing by shRNA transfection for 72 h, the bladder cancer 5,637 cells were treated with 100 ng/ml LTα1β2 for activation of LTβR. *P<0.05, compared with shRNA-C for each gene. Error bars represented the standard deviation. LTβR, lymphotoxin β receptor; LT, lymphotoxin; RelA, RELA proto-oncogene NF-κB subunit; TNFα, tumor necrosis factor αl; IL, interleukin; shRNA-C, control scramble short hairpin RNA; shRNA-T, LTβR-specific shRNA.

and protein level by RT-qPCR and western blot, respectively (Fig. 2). Compared with the control group, expression of LTβR in cells transfected with LTβR-specific shRNA was reduced by 65-75% at the mRNA level (P=0.001; Fig. 2A) and ~50% at the protein level (P<0.001; Fig. 2B). Compared with the shRNA-C group, RelA mRNA expression was downregulated by ~33% (P=0.012), TNFα by 27% (P=0.011) and IL-1β by 26% (P=0.011) in the shRNA-T group following activation of LTβR (Fig. 3).

Activation of LTβR has no effect on 5,637 cell growth despite increased mRNA expression of proliferation-related genes CyclinD1 and Survivin. There are contrasting observations regarding the role of LTβR signaling pathway in tumor cell apoptosis and growth promotion. In the present study, LTβR activation in 5,637 cells resulted in a 2.7-fold upregulation of CyclinD1 mRNA expression levels (P=0.002) and a 1.3-fold upregulation of Survivin mRNA expression levels (P=0.035), compared with control unstimulated cells (Fig. 4A). However, when cell viability was measured by CCK-8 assay, no significant change was observed in the % of cell viability in cells stimulated with LTα1β2 for 24 or 48 h, compared with control unstimulated cells (Fig. 4B).

Discussion

It is well documented that NF-κB signaling represents a critical link between inflammation and cancer. NF-κB signaling can be activated by a great variety of stimuli, including inflammatory mediators and stress response. The diversity of stimuli endows NF-κB signaling with a great complexity and multiplicity as to the biology processes it regulates. Activation of NF-κB signaling (usually assessed by the presence of nuclear RelA) has been observed in many types of cancer, including colon cancer, hepatocellular carcinoma, prostate cancer, pancreatic cancer, various types of leukemia and melanoma (18). In addition, it has been reported that LTβR is critical in NF-κB-dependent promotion of HCC (13) and prostate cancer (19). Higher expression levels of RelA and RelB have been observed in bladder cancer and bladder inflammation tissues, compared with normal tissues by RT-PCR, suggesting a potential link between NF-κB signaling and the development of bladder cancer (8). Thus, it was hypothesized that LTβR activation may enhance the expression levels of these main members of NF-κB signaling in bladder cancer cells. LTβR activation participates in both the canonical and noncanonical NF-κB signaling pathways (20). Notably, bladder cancer 5,637 cells exhibited a significant increase in RelA transcripts following LTα1β2 treatment compared with unstimulated cells, but only a non-significant trend toward increased RelB mRNA levels was observed. These findings suggested that the NF-κB canonical signaling member RelA may be the main target gene of LTβR activation.

It is estimated that underlying infections and inflammatory reactions are linked to 25% of all cancer cases (21). Infections with hepatitis B (HBV) or C (HCV) virus increase the risk of HCC (13), infections with *Schistosoma* or *Bacteroides*

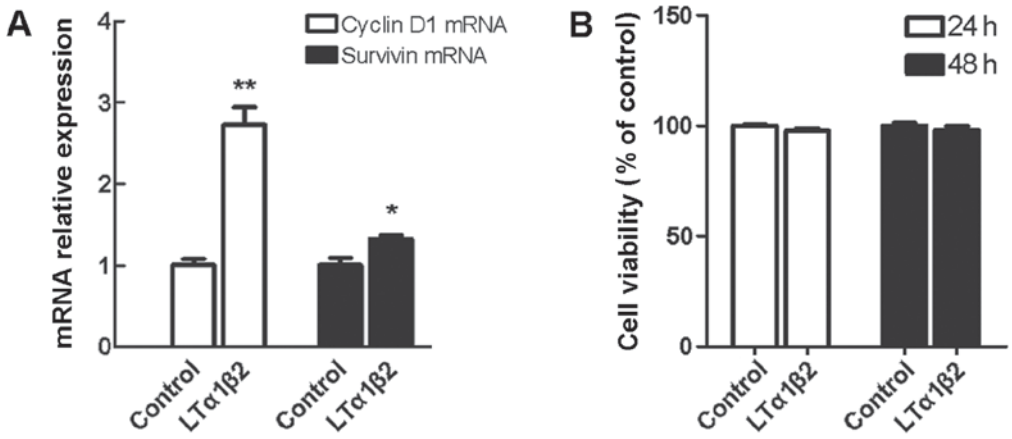

Figure 4. Effect of LTβR activation in bladder cell cell growth. LTβR was activated in 5,637 cells by 100 ng/ml LTα1β2 treatment. (A) mRNA expression levels of the proliferation-related genes CyclinD1 and Survivin. (B) Cell viability (as % of control cells) was examined at 24 and 48 h post-stimulation. *P<0.05 and **P<0.01, compared with control unstimulated cells. Error bars represented the standard deviation. LTβR, lymphotoxin β receptor; LT, lymphotoxin.

species are linked to bladder cancer and colon cancer, respectively (22,23), and inflammatory bowel disease (IBD) greatly increases the risk of colorectal cancer (24). It is now well established that the inflammatory microenvironment is important in the development of cancer, which is why it was added as the seventh hallmark of cancer (25). The inflammation present on the tumor microenvironment is characterized by infiltration of leukocytes (such as lymphocytes and neutrophils), inflammatory mediators (such as cytokines and chemokines) and persistent activation of molecular signaling pathways (such as NF-κB and protein kinase B signaling) (26). These elements in the microenvironment are often subject to a feed-forward loop; for example, activation of NF-κB in immune cells induces production of cytokines which then activate NF-κB in cancer cells, resulting in the release of chemokines that attract more inflammatory cells into the tumor tissue (27).

In the present study, activation of LTβR was demonstrated to promote the mRNA expression of cytokines TNFα and IL-1β. TNFα, IL-6 and IL-1β are target genes of NF-κB signaling (27), and overproduction of TNFα and IL-1β are stimuli in the persistent activation of NF-κB signaling (28). Popivanova *et al* (29) have reported that increased TNFα expression and increased numbers of infiltrating leukocytes expressing its major receptor, p55 (TNF-Rp55), are followed by the initiation and progression of colitis-associated colon carcinogenesis. Tu *et al* (30) have demonstrated that stomach-specific expression of human IL-1β in transgenic mice leads to spontaneous gastric inflammation and cancer, and this effect is dependent on early recruitment of myeloid-derived suppressor cells (MDSCs) to the stomach, which are activated by IL-1β in a IL-1R/NF-κB-dependent manner. Based on these previous observations, it is possible that the LTβR overexpression of TNFα and IL-1β observed in 5,637 bladder cancer cells may be involved in the persistent activation of NF-κB signaling, resulting in the feed-forward loop of chronic inflammation in bladder cancer. Further studies will be required to explore the underlying molecular links and mechanisms.

Activation of LTβR has been demonstrated to induce both tumor growth inhibition and promotion. By contrast, LTα1β2-induced activation of LTβR activates NF-κB to induce chronic inflammation. In HCC, sustained activation of LTβR signaling results in chronic inflammation response, which promotes HCC development in a NF-κB-dependent manner (13). It has also been reported that the expression of pro-angiogenic chemokine C-X-C motif ligand 2 is increased in mouse fibrosarcoma cells, paralleled by enhanced solid tumor growth, when stimulated with an agonistic anti-LTβR antibody (31). By contrast, LTβR activation effectively inhibits human colorectal tumor growth in a xenograft mouse model (9). LTβR directly mediates cytotoxic lymphocyte-directed tumor rejection (15,16). Hu *et al* (14) determined that LTβR mediates caspase-dependent tumor cell apoptosis in colon carcinoma, mammary carcinoma and sarcoma, and that LTβR-activated NF-κB potentially functions as a tumor suppressor. In the present study, activation of LTβR had no effect on bladder cancer cell growth, despite increasing the mRNA expression levels of proliferation-related genes CyclinD1 and Survivin. Since tumor development involves a variety of molecular signaling pathways and complex processes, a limitation in the present study was that only two proliferation-related genes were examined, and therefore the exact regulation mechanism and function of LTβR signaling in proliferation was not fully assessed. Further studies will be needed to investigate the molecular mechanism of LTβR in the regulation of cell proliferation.

In summary, the present study indicated a potential role of LTβR signaling in inducing expression of NF-κB canonical pathway members and pro-inflammatory mediators. Further studies are needed to investigate in greater detail the link between LTβR signaling and the biological processes leading to the development and progression of bladder cancer, potentially guiding in the future the development of novel targeted drugs therapies.

Acknowledgements

The present study was supported by Zhejiang Provincial Natural Science Foundation of China (grant no. Y2110555), Zhejiang Medical Technology and Education Foundation of China (grant no. 2016KYB200) and Wenzhou Science and Technology Plan Project (grant no. Y20140609).

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