

RESEARCH ARTICLE

Effect of TLR4 and B7-H1 on Immune Escape of Urothelial Bladder Cancer and its Clinical Significance

Yong-Hua Wang¹, Yan-Wei Cao¹, Xue-Cheng Yang¹, Hai-Tao Niu¹, Li-Jiang Sun¹, Xin-Sheng Wang¹, Jing Liu^{2*}

Abstract

Background/Aim: Toll-like receptor 4 (TLR4) and B7-H1, both normally expressed restricted to immune cells, are found to be aberrantly expressed in a majority of human tumors and may play important roles in regulation of tumor immunity. It has been shown that urothelial bladder cancer (UBC) patients can manifest tumoral immune escape which may be a potential critical factor in tumor pathogenesis and progression. However, so far, the mechanisms of UBC-related immune escape have not been clarified. The aim of this study was to investigate the effect of TLR4 and B7-H1 on immune escape of UBC. **Methods:** Bladder cancer T24 cells were pre-incubated with LPS and co-cultured with tumor specific CTLs. CTL cytotoxicity and apoptosis rates were measured by MTT assay and flow cytometry, respectively. The effects of an ERK inhibitor on B7-H1 expression and CTL cytotoxicity against T24 cells were also evaluated. In addition, TLR4, B7-H1 and PD-1 protein expression was analyzed by immunohistochemistry in 60 UBC specimens and 10 normal urothelia. **Results:** TLR4 activation protected T24 cells from CTL killing via B7-H1 overexpression. However PD98059, an inhibitor of ERK, enhanced CTL killing of T24 cells by reducing B7-H1 expression. TLR4 expression was generally decreased in UBC specimens, while B7-H1 and PD-1 were greatly overexpressed. Moreover, expression of both B7-H1 and PD-1 was significantly associated with UICC stage and WHO grade classification. **Conclusions:** TLR4 and B7-H1 may contribute to immune escape of UBC. Targeting B7-H1 or the ERK pathway may offer new immunotherapy strategies for bladder cancer.

Keywords: Toll-like receptor 4 - B7-H1 - urothelial bladder cancer - immune escape

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Introduction

Urothelial bladder cancer (UBC) is the most common malignancy of the urinary tract and the 7th most common cancer in men and the 17th in women (Babjuk et al., 2013). Although transurethral resection combined with intravesical chemotherapy has become the standard treatment for superficial UBC, approximately 50-70% of superficial UBC will relapse, with a 10-20% risk of progression to invasive tumor (Kaufman et al., 2009). Some studies have shown that UBC patients have tumor associated immune suppression and immune escape, which may contribute to the high recurrence rate and aggressive biological behavior (Herr et al., 1976; Mukamel et al., 1982). But the etiology of UBC-related immune escape remains unclear.

Toll-like receptor 4 (TLR4), which is thought to be restricted to immune cells, is a critical player in both innate and adaptive immunity. However, many studies have shown that TLR4 signaling can be usurped by tumor cells and trigger tumor self-protection mechanisms leading to immune escape (Huang et al., 2005). B7-H1 (also known

as PD-L1), which also normally expressed restrictedly in immune cells, is a newly discovered T-cell costimulatory molecule. It has been shown that B7-H1 can inhibit immune responses by inducing T-cell apoptosis, impairing cytokine production, and diminishing the cytotoxicity of activated T cells and may endow tumors with a mechanism to escape host immune destruction (Dong et al., 2002; Iwai et al., 2002; Blank et al., 2004). Recently, a variety of human tumor cell lines and freshly isolated human tumors have now been reported to aberrantly express TLR4 and B7-H1 (Dong et al., 2002; Huang et al., 2005). Our previous study showed that TLR4 and B7-H1 were constitutively expressed on T24 cells, which were derived from a patient with UBC. Furthermore, TLR4 activation by LPS up-regulated B7-H1 expression in a concentration and time dependent manner (Gong et al., 2013). Qian et al also demonstrated that MAPK pathway, especially ERK pathway was involved in LPS-induced B7-H1 expression in bladder cancer cells and inferred that blocking of B7-H1 or ERK pathway may be new targeted molecular strategies for bladder cancer (Qian et al., 2008). All these results implied the associations of TLR4 and B7-H1 with UBC-

¹Department of Urology, ²Department of Pediatrics, The Affiliated Hospital of Qingdao University, Qingdao, China *For correspondence: doctorliujing@sina.com

related immune escape. However, the specific effect of LPS-induced TLR4 activation and B7-H1 expression on CTL-mediated immune response in bladder cancer and, perhaps more important, the question whether blocking B7-H1 or ERK pathway can inverse the immune escape effect are not well understood.

Therefore, in this study we investigated the effect of LPS-induced TLR4 activation and B7-H1 expression on functionality of CTLs killing against bladder cancer cells. We further tested whether ERK inhibitor could inhibit B7-H1 expression and restore sensitivity of bladder cancer cells to CTLs. Moreover, we also studied TLR4, B7-H1 and PD-1 protein expressions in UBC specimens and analyzed the relationship between those expressions and clinicopathological features.

Materials and Methods

Cell culture

Human bladder cancer T24 cells were obtained from China Center for Type Culture Collection (CCTCC). Cells were cultured in RPMI 1640 with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

LPS stimulation and drugs intervention

Cultured T24 cells were treated with LPS (*E. coli* 0111:B4, Sigma-Aldrich), which was used to activate TLR4 signaling. Blockade of B7-H1 on T24 cells was accomplished by incubating with B7-H1 blocking antibody (clone MIH1, eBioscience, USA) and PD98059 (Sigma-Aldrich, USA) was used as an inhibitor of ERK. T24 cells were pre-incubated with MIH1 or PD98059 for 2 h before the CTL assay.

Generation of tumor specific CTLs

Normal human peripheral blood was obtained from volunteer donors with informed consent and the study protocol was approved by the ethics committee of the affiliated hospital of Qingdao university. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation and then cocultured in 24-well plates with mitomycin treated T24 tumor cells at a ratio of 10:1. Cell cocultures were maintained in complete media with recombinant human interleukin-2 (Peprotech, USA, 100 units/mL) and viable lymphocytes were harvested 5 days later as tumor specific CTLs.

CTLs cytotoxicity and apoptosis assays

CTLs cytotoxicity against T24 cells was estimated by MTT assay. LPS-stimulated T24 cells were seeded in 96 well plates and used as targets for CTLs at an effector/target ratio of 20:1. 20 µl MTT was added in each well and the resultant formazan crystals were dissolved in 150 µl dimethyl sulfoxide. The absorbance of each well was measured with a microplate reader at 570 nm and each assay was repeated at least three times.

To assay for apoptosis of CTLs, tumor specific CTLs were cocultured with LPS-stimulated T24 cells for 5 days. Cells were then harvested and stained with

annexin V (Immunotech, France) and propidium iodide (PI, Immunotech, France) for 1 h and the samples were analyzed by flow cytometry (FACSCalibur, Becton Dickinson) and CellQuest software (BD Biosciences). Apoptosis was calculated as the percentage of annexin V+ PI- cells in the viable cell fraction.

Western blot

Total proteins from cultured cells were separated on polyacrylamide gel, transferred onto nitrocellulose membrane and incubated with anti-B7-H1 polyclonal antibody (Santa Cruz, USA) overnight at 4°C. Membranes were then washed 3 times in washing buffer and incubated with anti-rabbit IgG conjugated to peroxidase for 1 hour at room temperature. Immunoreactivity was determined by chemiluminescence (Santa Cruz, USA) according to manufacturer instructions.

Clinical specimens

Specimens were collected from 60 patients with UBC who underwent surgical resection at the Affiliated Hospital of Qingdao University (Qingdao, China) between 2009 and 2012. Besides, 10 samples from normal urothelium were collected and served as a control group. Written informed consent was obtained from the patients and the study protocol was approved by the ethics committee of the affiliated hospital of Qingdao university. Study exclusion criteria were: bladder infection before surgery, preoperative radiation therapy or chemotherapy, and inadequate tissue for immunohistochemical evaluation. All slides of all tumors were reviewed by an experienced pathologist. Tumor stage and grade were defined according to the Union Internationale Contre le Cancer (UICC) and World Health Organization (WHO) criteria. All clinicopathologic parameters are summarised in Table 1.

Immunohistochemistry

Immunohistochemical staining was performed on 4-µm thick sections of the tissue microarray blocks. Paraffin-embedded sections were mounted on superfrost glass slides, deparaffinized, rehydrated in a graded ethanol series, and then subjected to microwave antigen retrieval. Endogenous peroxidase activity was blocked by using 3% hydrogen peroxide. Sections were incubated for 2h at room temperature with anti-TLR-4, anti-B7-H1 and anti-PD-1 polyclonal antibody respectively (Boster, China). Immunohistochemical staining was then performed according to the instruction of PV-6000 two-step immunohistochemical method. Sections were then counterstained with hematoxylin and then dehydrated, cleared and mounted. Tumors were considered positive for TLR4 and B7-H1 if there was histologic evidence of cell plasma membrane staining in 10% or more of cells. Cases with <10% tumor staining were considered negative. To evaluate the staining of PD-1 on tumor-infiltrating lymphocytes (TILs), numbers of positive TILs in five randomly selected areas were scored and the median was recognized as the dividing value of positive and negative.

Statistical analysis

Statistical analysis was performed using SPSS 17.0.

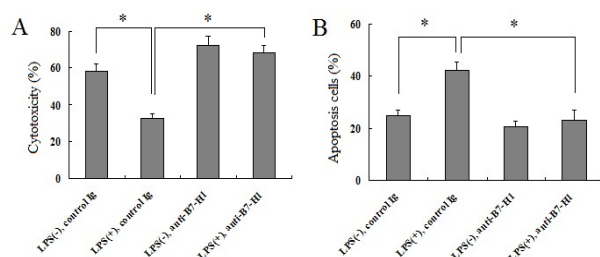


Figure 1. Effect of TLR4 Activation and B7-H1 Expression in T24 Cells on CTLs Cytotoxicity and Apoptosis. Before the CTL assay, T24 cells were pre-incubated with or without LPS (1 µg/ml), anti-B7-H1 mAb (10 µg/ml) or control Ig. CTLs cytotoxicity against T24 cells was estimated by MTT assay (A) and CTLs apoptosis rate was measured by flow cytometry (B). The data presented were generated from three independent experiments (*indicates significant differences)

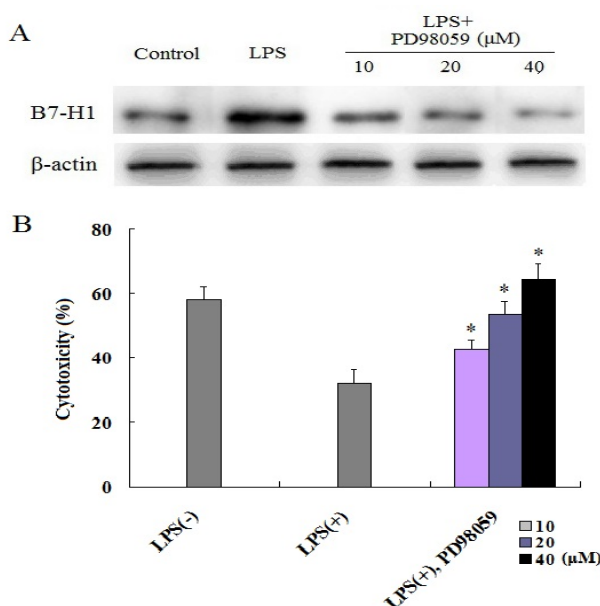


Figure 2. Effect of ERK Inhibitor on B7-H1 Expression and CTLs Cytotoxicity. T24 cells were pretreated with or without LPS (1 µg/ml), the indicated concentrations of PD98059 (10 µM, 20 µM, 40 µM). B7-H1 protein expression was measured by western blot (A) and CTLs cytotoxicity against T24 cells was estimated by MTT assay (B). The data presented were generated from three independent experiments (*indicates significant differences between LPS and LPS plus PD98059)

Data were obtained and enumerated by t-test and chi-square test, respectively. Spearman rank association test was used to analyze the relevance of TLR4, B7-H1 and PD-1 expression in UBC. $P < 0.05$ was regarded as statistically significant.

Results

TLR4 activation protects T24 cells from CTLs killing via B7-H1 overexpression

To evaluate the significance of TLR4 activation and B7-H1 expression in T24 cells, we studied T24 cells cocultured with tumor specific CTLs and then tested CTLs cytotoxicity and apoptosis rate. Under basal conditions, blocking B7-H1 with a specific antibody had little effect on CTLs cytotoxicity and apoptosis rate. However, when T24 cells were pre-incubated with LPS, they demonstrated

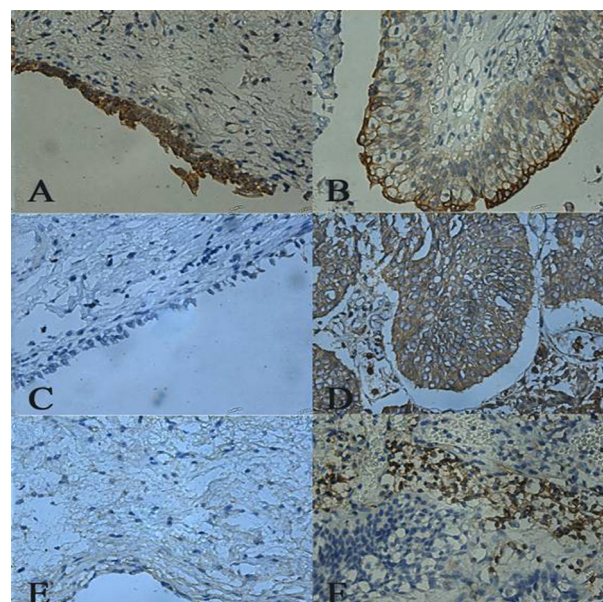


Figure 3. TLR4, B7-H1 and PD-1 Protein Expression in Normal Urothelium and UBC. Representative slides (×400) of TLR4 protein staining in normal urothelium (A) and UBC tissue (B); B7-H1 protein staining in normal urothelium (C) and UBC tissue (D); PD-1 protein staining in normal urothelium (E) and UBC tissue (F)

a higher resistance to CTL-mediated killing and increased the apoptosis rate of CTLs. However, this protection was partially abolished when B7-H1 blocking antibody was added to the medium. Thus, TLR4 activation in T24 cells can protect them from CTL killing via B7-H1 overexpression (Figure 1).

ERK inhibitor enhances CTLs killing against T24 cells by reducing B7-H1 expression

Previous studies demonstrated that MAPK pathway, especially ERK pathway was involved in LPS-induced B7-H1 expression in bladder cancer cells. PD98059, an inhibitor of ERK, was used in this study to test whether ERK inhibitor could inhibit B7-H1 expression and restore sensitivity of bladder cancer cells to CTLs. We found that B7-H1 protein expression, which was inducible with LPS, was significantly reduced in T24 cells after exposure to PD98059. Furthermore, pre-incubation with PD98059 resulted in increased CTLs killing against T24 cells. Thus, we confirm that ERK is an important regulator of B7-H1 expression in T24 cells and ERK inhibitor enhances CTLs killing by reducing B7-H1 expression (Figure 2).

TLR4, B7-H1 and PD-1 expressions in UBC specimens

To evaluate the expressions and significance of TLR4 and B7-H1 in UBC specimens, TLR4, B7-H1 and PD-1 protein expressions were analyzed from 60 UBC specimens and 10 normal urotheliums by immunohistochemistry. We found that TLR4 was expressed in all normal urotheliums compared to generally decreased in UBC specimens (41/60, 68.3%). Expressions of B7-H1 and its receptor PD-1 in normal urotheliums were not observed. However, B7-H1 immunostaining was noted in 43/60 (71.7%) of UBC specimens and PD-1 immunostaining was detected on TILs in 48/60 (80.0%) of UBC specimens (Figure 3).

Table 1. Association of TLR4, B7-H1 and PD-1 Expressions with Clinicopathological Features of UBC

		N	TLR4(%)	P	B7-H1(%)	P	PD-1(%)	P
Age (y)				0.121		0.832		0.896
<65	26	57.7			73.1		80.8	
≥65	34	76.5			70.6		79.4	
Gender				0.175		0.299		0.195
Male	46	73.9			76.1		84.8	
Female	14	50.0			57.1		64.3	
Tumor grade				0.018		0.036		0.013
Low	26	84.6			57.7		65.4	
High	34	55.9			82.4		91.2	
Tumor stage				0.004		0.024		0.020
NMIBC	32	84.4			59.4		68.8	
MIBC	28	50.0			85.7		92.9	

Association of TLR4, B7-H1 and PD-1 expressions with clinicopathological features of UBC

According to the UICC and WHO criteria, 60 specimens were classified as low grade/high grade group and NMIBC/MIBC group. TLR4, B7-H1 and PD-1 expressions had no association with gender or age. Interestingly, we found that TLR4 expression was decreased in low grade group or NMIBC group than in normal urothelium. In high grade group or MIBC group, the decrease in TLR4 expression was more pronounced. Tumor specimens from patients with high grade group or NMIBC group showed significantly higher expressions of B7-H1 and PD-1. Moreover, PD-1 expression on TILs had a significant correlation with B7-H1 expression in tumor cells (Table 1).

Discussion

UBC has been characterized as an immunogenic cancer that contains large amounts of TILs and is sensitive to immunotherapy with BCG (Tsujihashi et al., 1989; Patard et al., 1998). However, it has been shown that UBC patients can manifest acquired tumor immune dysfunction, particularly affecting lymphocytes. Circulating T cells from UBC patients have been found to be unresponsive to polyclonal T-cell activation compared with healthy donor cells (Luskog et al., 2007). These evidences indicate that tumor immune escape may be a potentially important mechanism for pathogenesis and progression of UBC.

TLR4 and B7-H1 are both critical molecules in the initiation and regulation of immune responses. TLR4 is a member of the Toll-like receptor family and has been reported as LPS signaling receptor in urinary tract infection, which is consistent with the high incidence of gram-negative bacteria containing LPS as urinary tract infectious agents (Zhang et al., 2004; Song et al., 2008). The roles of TLR4 in tumor immunity remain controversial. Previous studies showed that TLR4 could drive DC maturation and recruit lymphocyte to tumors which were beneficial to induce tumor immunity. However, Huang et al demonstrated that activation of TLR4 signaling in tumor cells could facilitate evasion of immune surveillance and inhibition of TLR4 function in tumors might be beneficial to the host (Huang et al., 2005). B7-H1 is a cell-surface glycoprotein belonging to the B7

family that can inhibit immune responses by binding to its receptor PD-1 on the surface of T lymphocytes and consequently inducing antigen-specific T-cell apoptosis or anergy. Tumor cell expression of B7-H1 has been shown to endow tumors with a mechanism to escape from host antitumor immunity (Dong et al., 2002). Here, we described that TLR4 activation protected T24 cells from CTLs killing by up-regulation B7-H1 expression. Although the endogenous ligand for TLR4 is not clear, there is evidence for the existence of endogenous TLR4 ligands including heat shock protein 70 and beta-defensin 2 in tumor microenvironment (Biragyn et al., 2002; Vabulas et al., 2002). So we support the idea that TLR4 may act as a double-edged sword, enhancing host immunity against the tumor by stimulating antigen presenting cells, and protecting tumor cells from immune surveillance. Our data suggest that TLR4-signaling pathway may participate in the immune escape process of bladder cancer by up-regulation B7-H1 expression. And this may be a potentially important mechanism which contributes to UBC-related immune escape and tumor progression.

It has been demonstrated that B7-H1 expression is activated by IFN- γ and TLR ligands via a common signaling pathway involving MyD88/MEK/ERK/STAT1, which activates transcription (Liu et al., 2007). Previous studies showed that MAPK pathway, especially ERK pathway was involved in LPS-induced B7-H1 expression in bladder cancer cells (Qian et al., 2008). By using specific inhibitors, we studied the effect of blocking ERK pathway on B7-H1 expression and CTLs killing against T24 cells. Our data confirmed that ERK inhibitor not only inhibited B7-H1 expression, but also restore sensitivity of T24 cells expressing B7-H1 after stimulation with LPS to CTL-mediated killing. Interestingly, ERK inhibitors including PD98059 are currently under clinical development for the treatment of various types of tumors. They can inhibit tumor cell proliferation or induce cell death by blocking growth and survival signals. While our data show that ERK inhibitors can also kill tumor cells indirectly by suppression of B7-H1 expression and CTL-mediated killing. Therefore we suggest that targeting ERK pathway or B7-H1 may become new molecular therapy strategies for bladder cancer.

Recently, a lot of human tumors have now been reported to aberrantly express TLR4 and B7-H1 (Dong et al., 2002; Huang et al., 2005). Here, we also detected TLR4, B7-H1 and PD-1 protein expressions in UBC specimens and analyzed the relationship between those expressions and clinicopathological features. Although TLR4 expression in UBC specimens was decreased, most NMIBC still expressed TLR4 at a sizable level. Ayari et al also observed the changes of TLR expressions detected in bladder cancer cell lines and tumor specimens by immunohistochemistry and they attributed this difference to the cell culture process (Ayari et al., 2011). Further mechanistic studies are needed to explore the reasons of decreased TLR4 protein expressions in UBC specimens. Moreover, we observed that B7-H1 and PD-1 were greatly overexpressed in UBC specimens. B7-H1 and PD-1 expressions were both significantly associated with UICC

stage and WHO grade classification. As both UICC stage and WHO grade are recognized as important prognostic factors in UBC, B7-H1 and PD-1 expressions may be associated with UBC prognosis. Thus, our data encourage further investigations to determine the prognostic value of B7-H1 and PD-1 in UBC.

Currently, increasing appreciation of TLR4 and B7-H1 in the modulation of immune response resulted in the development of new strategies for cancer immunotherapy. TLR4 antagonists have been developed for the treatment of several cancers and antibodies blockade of B7-H1 have been shown to potentiate antitumoral immunity in vitro and in vivo (Sun et al., 2008; Okudaira et al., 2009; Hasan et al., 2011; Elhag et al., 2012). Moreover, it is well known that TLR4 may involve in the response to BCG during BCG immunotherapy for bladder cancer. But recently Inman et al reported that the majority of UBC that failed BCG immunotherapy exhibited extremely intense B7-H1 expression within the BCG granulomas found in proximity to their recurrent tumors (Inman et al., 2007). It indicated that the longitudinal accumulation of B7-H1-expressing cells within and around BCG-induced granulomata might inhibit T-cell interactions with relevant APCs, or responses directed against tumor or pathogenic antigens, to ultimately abrogate the effectiveness of BCG immunotherapy. Therefore, we suggest that TLR4 and B7-H1 may be important factors promoting the eventual loss of BCG effectiveness over time. But this hypothesis remains untested and needs further investigations.

In conclusion, we have demonstrated that TLR4 activation protects T24 cells from CTLs killing by up-regulation B7-H1 expression. While blocking B7-H1 or ERK pathway can restore sensitivity of T24 cells to CTL-mediated killing. These results suggest that TLR4 and B7-H1 may contribute to the immune escape of UBC. Further in vitro and in vivo studies are warranted to elucidate the functions of TLR4 and B7-H1 in bladder cancer, which may provide new therapeutic targets in bladder cancer immunotherapy.

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