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Expression and Functional Analysis of Toll-like Receptor 4 in Human Cervical Carcinoma

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Abstract Toll-like receptors are expressed in human immune cells and many tumors, but the role of toll-like receptor 4 (TLR4) in the development of tumors is controversial. We demonstrated the expression, distribution, and functional activity of TLR4 in tissues of normal cervix, cervical intraepithelial neoplasia (CIN), invasion cervical cancers (ICC), and different human papillomavirus (HPV)-infected cervical cancer cells. The results showed that TLR4 expression was in accordance with the histopathological grade: higher in ICC than in CIN, and low in normal cervical tissues and malignant cervical stroma. Expression was higher in SiHa (HPV16+) than in HeLa (HPV18+) cells, but was not observed in C33A (HPV−) cells. After treatment with its agonist, lipopolysaccharide (LPS), the expression levels of TLR4 was increased and apoptosis resistance was induced in SiHa cells, but not in HeLa or C33A cells. Meanwhile, LPS treatment did not alter the cell cycle distribution in SiHa cells. The mechanism of apoptosis resistance may be related to HPV16 infection and not correlated with the cell cycle distribution. Targeting TLR4 in combination with traditional drug treatment may serve as a novel strategy for more effectively killing cancer cells.

Keywords Toll-like receptor 4 · Cervical cancer · Cervical intraepithelial neoplasia · HPV · Lipopolysaccharide

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Introduction

Cervical cancer, the third most commonly diagnosed cancer, is primarily caused by human papillomavirus (HPV) infections (Jemal et al. 2011). The healthy vagina contains normal endogenous bacterial flora, and the cervix is exposed to various bacteria in the environment. Moreover, bacterial vaginosis, mainly caused by the Gram-negative bacteria *Gardnerella vaginalis*, is the most common vaginal disorder among women of reproductive age. However, the potential effects of bacteria in the female reproductive tract on the development of cervical intraepithelial neoplasia (CIN) and cervical carcinomas have not been thoroughly explored.

Inflammation is an adaptive response that is triggered by a variety of abnormal conditions, including infection and tissue injury as well as subtler alterations of tissue homeostasis, and chronic inflammation may increase the risk for tumorigenesis (Mantovani et al. 2008). Emerging evidence suggests that Toll-like receptors (TLRs) are expressed on immune cells and on many cancer cells. TLRs may participate in carcinogenesis and tumor progression during chronic inflammation by shaping the tumor microenvironment. TLR signaling activated by certain ligands may considerably induce the secretion of proinflammatory cytokines, such as cyclooxygenases, chemokines, VEGF, matrix metalloproteinases, IL-6, IL-12, and

tumor necrosis factor-α (TNF-α). These cytokines regulate the tumor microenvironment and simultaneously promote malignant transformation of epithelial cells, immune escape, apoptosis resistance, and chemoresistance (Goto et al. 2008; He et al. 2007; Fukata et al. 2007; Yu and Chen 2008). Current studies are mainly focused on TLR5 and TLR9 (Kim et al. 2008; Hasan et al. 2007); however, the biological functions of TLRs in tumorigenesis remain unclear.

Toll-like receptor 4 (TLR4) is an important transmembrane pattern recognition receptor that recognizes exogenous ligands, such as lipopolysaccharide (LPS), as well as endogenous ligands, such as heat shock proteins (HSP60, HSP70), high mobility group box 1 (HMGB1), and various products of the extracellular matrix (Ohashi et al. 2000; Vabulas et al. 2002; Park et al. 2006). In some diseases, such as alcohol-induced neuroinflammation and brain damage, TLR4 deficiency protects against ethanol-induced glial activation, induction of inflammatory mediators, and apoptosis (Alfonso-Loeches et al. 2010). TLR4 shows increased expression in brain tissues in Alzheimer's disease (Walter et al. 2007), regulates the inflammatory response, and affects the tumor microenvironment by recognizing endogenous and exogenous ligands (Andersen et al. 2006). Stimulation of TLR signaling on endocervical epithelial cells can induce the secretion of proinflammatory cytokines, such as TNF-α, IL-6, and IL-12 (Donders et al. 2003; Zariffard et al. 2005; Huang et al. 2005), which can interfere with virus infection (Bachmann et al. 2002; Soto et al. 1999). However, there are controversies over the expression of TLR4 in primary endocervical epithelial cells and cervical cancer cells (Fichorova et al. 2002). We detected the expression and location of TLR4 in normal cervical tissues, CIN, invasive cervical carcinomas (ICC), and different HPV-infected cervical cancer cell lines. We tested the change in TLR4 expression, apoptosis rate, and cell cycle distribution after LPS treatment. In this way, we evaluated the influence of HPV infection on TLR4 expression and functions and the role of TLR4 in the initiation and development of cervical carcinoma in vitro.

Materials and Methods

Tissues Samples and Cervical Cancer Cell Lines

Paraffin-embedded, formalin-fixed tissue specimens diagnosed from January 2006 to December 2009 were used and included CIN I (30 cases), high-grade CINs (CIN II&III, 30 cases), invasive squamous cell carcinomas (29 cases), adenocarcinomas (20 cases), and normal cervical tissues (12 cases) from women who underwent hysterectomy for benign disease. All specimens were obtained from Yantai YuHuangDing Hospital, Qingdao University of Medicine and Tongji Hospital, Tongji Medical College. Two dedicated pathologists reviewed all cases with regard to histological type, grading, and lymph node status.

The human cervical cancer cell lines SiHa (HPV16-positive), HeLa (HPV18-positive), and C33A (HPV-negative) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle Medium containing 10 % fetal bovine serum at 37 °C in 5 % CO_2.

Immunohistochemical Studies

Sections (5-mm) were mounted on poly-L-lysine-coated glass slides and dried at 37 °C overnight, deparaffinized in xylene, washed in graded ethanol, and finally in phosphate-buffered saline (PBS, pH 7.4). Endogenous peroxidase activity was blocked with 3 % H_2O_2 for 15 min, and non-specific binding was blocked with normal goat serum for 30 min. Primary antibody (mouse anti-human TLR4, 1:400 dilution, Imgenex, Sorrento, CA) was applied at 4 °C overnight, and antigen–antibody complexes were detected according to streptavidin-peroxidase method (Vectastain ABC-kit, Vector Laboratories, Burlingame, CA). TLR4 immunoreactivity was determined using the well-standardized H-Score system ($H = I \times P$), as in our previous study (Ye et al. 2010). In this equation, I is the staining intensity, which was evaluated according to the following criteria: 0, no staining; 1+, weak intensity; 2+ moderate intensity; 3+, strong intensity. P is the percentage of positively stained cells in each sample. An H score ≥ 20 was considered positive.

Immunofluorescence Analysis

Cells were cultured on slides to approximately 70 % confluence, fixed with 4 % paraformaldehyde, and permeabilized with methanol. The slides were incubated with blocking solution (0.5 % BSA in PBS) and exposed to primary antibody (1:100 dilution) at 4 °C overnight and a fluorescein isothiocyanate/tetramethyl rhodamine isothiocyanate-conjugated secondary antibody. Morphological alterations in the cells were observed using a confocal laser scanning microscope (OLYMPUS, Japan).

Quantitative Real-time PCR Analysis of TLR4 mRNA Expression

Total RNA was extracted from cells using Trizol (Invitrogen). Real-time PCR was performed using the SYBR Green PCR Master Mix (Sigma) on an ABI Prism 7000. Two micrograms of total RNA was reverse transcribed into cDNA and amplified.

The specific primer pairs used were as follows: TLR4: sense (5'-TGA GCA GTC GTG CTG GTA TC-3'), antisense (5'-CAG GGC TTT TCT GAG TCG TC-3'); and GAPDH: sense (5'-AGA AGG CTG GGG CTC ATT TA-3'), antisense (5'-AGG GGC CAT CCA CAG TCT TC-3'). The real-time PCR conditions were 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. All reactions were run in triplicate, and the fold amplification of genes was determined by calculating the $2^{-\Delta\Delta Ct}$.

The PCR reaction mixtures were subjected to 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Each PCR product was analyzed by electrophoresis on a 1.5 % agarose gel containing 0.5 % ethidium bromide.

Western Blotting Analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % NonidetP-40, 1 % Triton X-100, 0.2 % SDS, 1 % sodium deoxycholate, 5 mM iodoacetamide, proteinase inhibitor cocktail, and 2 mM PMSF). Protein (50 μg) was denatured in SDS sample buffer at 100 °C for 10 min, separated by electrophoresis on a 10 % SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was blocked in TBST (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, and 0.05 % Tween 20) with 5 % non-fat milk for 1 h at 37 °C and then incubated with the primary antibodies (Santa Cruz rabbit anti-TLR4, 1:500; Santa Cruz rabbit anti-β-actin, 1:1000) in blocking buffer at 4 °C overnight and secondary antibody (Santa Cruz alkaline phosphatase-conjugated goat IgG, 1:1000) for 2 h at room temperature. Proteins were visualized with NBT/BCIP/buffer (1:1:50, Roche).

Flow Cytometry

SiHa, HeLa, and C33A cells treated with 0, 0.5, 1, 3, 5, and 10 μg/μL LPS (Sigma Chemical Inc., USA) for 24 h, were harvested, fixed in ice-cold 70 % ethanol, and stored at −20 °C overnight. All samples were washed once in PBS and resuspended in a solution containing propidium iodide (5 mg/mL) and RNase A (0.5 mg/mL) in PBS for an additional 30 min. Cells were sorted using a FACScan (BD Biosciences, USA) and analyzed with CellQuest version 3.3 software.

Statistical Analysis

Data analysis and statistics were performed using the SPSS statistical 13.0 software program. The data are presented as the mean ± SD of at least three experiments. Significant differences were estimated by Student's t test and the χ^2 test. $P < 0.05$ was considered statistically significant.

Results

The Expression Levels of TLR4 in Normal Cervical Epidermal Tissues, CIN, and ICC

We carried out immunohistochemical staining to confirm the association of TLR4 expression with clinical-pathological parameters. As summarized in Figure 1 and Table 1, TLR4 expression was positively correlated with histopathological grade in the following order: normal cervical epidermal tissues <low-grade CIN < high-grade CIN < ICC ($P < 0.01$). The positive expression rates of TLR4 in normal cervical epidermal tissues, CIN I, CIN II & III, and ICC were 8.33, 20.00, 26.67, and 55.10 %, respectively ($P < 0.005$). In normal cervical tissues, TLR4 was expressed weakly in the epidermal cell layer and negatively in stromal tissues (Fig. 1a). In cervical cancer tissues, a significant difference ($P < 0.01$) in positive staining was observed between samples with lymph node metastasis (76.92 %) and those without lymph node metastasis (47.22 %). Although TLR4 immunoreactivity increased gradually from cervical cancer stage I (50.00 %) to stages III & IV (62.50 %), there was no significant difference ($P > 0.05$). Additionally, no correlation was found between TLR4 expression and age or cell differentiation. These data suggested that in normal cervical epidermal tissues with low TLR4 expression, TLR4 contributes to the mucosal immunology in CINs and cervical cancer tissues with high TLR4 expression.

TLR4 Expression in Cervical Cancer Cell Lines

We tested the TLR4 expression and localization in cervical cancer cell lines using immunofluorescence, RT-PCR, and Western blot analysis. As shown in Fig. 2a,b TLR4 was located in the plasma membrane. SiHa cells displayed much stronger fluorescence than HeLa cells, and no fluorescence was detected in C33A cells. As shown in Fig. 2c, SiHa and HeLa cells had positive TLR4 expression at the mRNA level, but there was no statistical significance between the levels observed in these cell lines ($P > 0.05$). No TLR4 mRNA expression was detected in C33A cells. As shown in Fig. 2d, TLR4 protein expression is high in SiHa cells, but weak in HeLa cells. No TLR4 protein expression was detected in C33A cells.

The above-mentioned data demonstrated that SiHa cells had higher expression than HeLa and C33A cells, which suggests that TLR4 expression in cervical cells may be associated with HPV16 infection.

The Effect of LPS on TLR4 Expression

Previous studies have suggested that cancer cells are hyporesponsive to LPS at doses of 10–100 μg/mL for 24 h

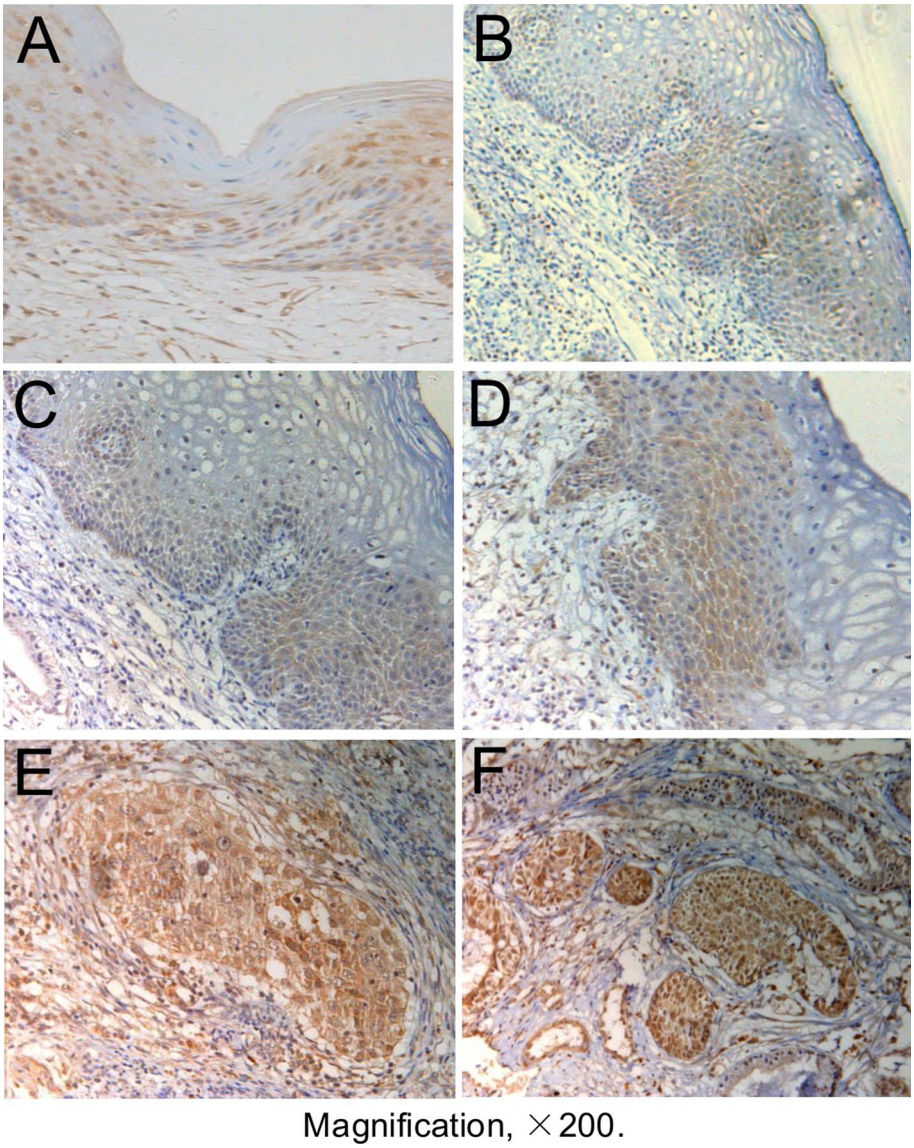

Fig. 1 Expression and distribution of TLR4 in cervical tissues. Immunohistochemical staining was performed in normal cervical epidermal tissues (**a**), CIN I (**b**), CIN II (**c**), CIN III (**d**), invasive squamous cell carcinoma (**e**), and adenocarcinoma of the cervix uteri (**f**). Original magnification, ×200

(MacRedmond et al. 2005). This was not the case in our study. With 10 and 20 μg/mL LPS for 24 h, TLR4 expression did not change a great deal in the cervical cancer cells (not shown); therefore, we chose doses that have not been previously reported. After using 0, 25, 50, 100, 150, and 200 μg/mL LPS for 24 h, we used real-time PCR to detect the TLR4 expression levels to test the relationship between TLR4 expression and HPV infection. All cells were stained with propidium iodide to ensure that the cell viability was 90–98 % (not shown).

In SiHa cells, TLR4 expression increased at low concentrations of LPS, especially at 25 μg/mL and 50 μg/mL LPS treatment ($P < 0.01$) (Fig. 3a), but declined when LPS was increased to 100 μg/mL. In HeLa cells, TLR4 expression decreased at low concentrations of LPS ($P < 0.05$), and when more LPS was added, TLR4 expression changed in an irregular way (Fig. 3b). In C33A cells, LPS had no effect on TLR4 expression (Fig. 3c).

Together, TLR4 was expressed at the highest level (Fig. 2) and had a positive reaction to LPS in SiHa cells. HeLa cells had a negative reaction, and C33A cells had no reaction to LPS. We concluded that TLR4 expression might cooperate with HPV16 in the development of cervical carcinoma.

The Effect of LPS on Apoptosis and Cell Cycle Distribution

SiHa, HeLa, and C33A cells were stained with Annexin V/PI to test for apoptosis and with PI only for cell cycle distribution analysis by flow cytometry after treatment with 25 μg/mL LPS.

Table 1 Expression of TLR4 in cervical tissues of different pathological changes and its correlation with clinical characteristics

Factor	No. of cases	TLR4 expression		Positive percentage (%)	$\chi^2(P)^a$
		Positive	Negative		
Normal	12	1	11	8.33	16.396
CIN					(<0.005*)
I	30	6	24	20.00	11.88
II&III	30	8	22	26.67	
ICC	49	27	22	55.10	(<0.01*)
Tumor stage					
I	20	10	10	50.00	0.423
II	21	12	9	57.14	
III &IV	8	5	3	62.50	(>0.05)
Nodal status					
Positive	13	10	3	76.92	8.45
Negative	36	17	19	47.22	(<0.01*)
Age(y)					
<40	19	9	10	47.37	0.327
≥40	30	18	12	60.00	(>0.05)
Cell differentiation					
High	10	4	6	40.00	1.602
Medium	17	9	8	52.94	
Low	22	14	8	63.64	(>0.05)

>0.05 indicated differences without statistical significance

<0.005* indicated significance differences of TLR4 expression in normal cervical epithelium, CINI, II&III and ICC samples

<0.01* indicated significance differences of TLR4 expression in CINI, II&III and ICC and in different nodal status samples

As shown in Figure 4a–d, the apoptosis rate decreased from 15.60 ± 1.21 % to 6.99 ± 0.75 % ($P < 0.05$) in SiHa cells and from 14.90 ± 1.53 % to 8.73 ± 1.40 % ($P < 0.05$) in HeLa cells, which showed TLR4-induced apoptosis resistance. In C33A cells, the apoptosis rate increased from 13.10 ± 1.06 % to 23.20 ± 1.47 %, possibly due to the slight toxicity of exogenous LPS.

From the data in Fig. 5 and Table 2, only the G0/G1 and G2/M arrest in HeLa cells was statistically significant with 25 μg/mL LPS treatment ($P < 0.05$). The cell cycle did not change at other concentrations of LPS (data not shown).

Collectively, TLR4 stimulates apoptosis resistance, especially in HPV16+ cervical cancer cells. TLR4 expression may interact with HPV16 infection in the progression of cervical cancer. LPS had no effect on cell cycle distribution, so we must further explore the mechanisms of apoptosis resistance.

Discussion

Although invasion and metastasis are distinguishing features of malignancy and the main reasons for mortality, HPV infections are the principal cause for the development and aggravation of ICC (Jemal et al. 2011). However, the cervix is frequently exposed to various floras other than HPV. As new methods emerge and many genes related to immune function are detected, much attention is being paid to the effects of the tumor environment. Activated TLR signaling on epithelial cancer cells may induce malignant transformation, tumor immune escape, apoptosis resistance, and chemoresistance (Yu and Chen 2008). The normal reproductive genital tract flora as well as bacterial vaginosis expose the cervix to TLR ligands; therefore, we hypothesized that vaginal bacterial conditions may be involved in the initiation and development of cervical carcinoma by TLRs.

TLR4 is expressed in some epithelial cells and plays an essential role in the defense against microbes by recognizing conserved bacterial molecules (Wolfs et al. 2002; Mukherjee et al. 2008; Schmausser et al. 2004; Pivarcsi et al. 2003). However, because of different races and living environments, there are controversies about the expression of TLR4 in epithelial cells. (Mempel et al. 2003) have found that TLR4 is not expressed in human keratinocytes. The components from vaginal bacterial conditions can induce proinflammatory cytokine secretion, such as TNF-α, IL-6, and IL-12, by stimulating TLR4 on endocervical epithelial cells (Donders et al. 2003; Zariffard et al. 2005; Huang et al. 2005). These proinflammatory cytokines can interfere with virus infection. TNF-α secreted in response to the stimulation of cervical epithelial cells may block the transcription of HPV DNA and cause several effects in HPV-immortalized cells (Bachmann et al. 2002; Soto et al. 1999). Other studies have found that the expression of TLR4 declines progressively along the reproductive tract, with the highest expression in the upper reproductive tract mucosa, and was barely detectable or absent in the ectocervix and vagina (Fazeli et al. 2005; Pioli et al. 2004). Fichorova et al. did not detect the expression of TLR4 in epithelial cells from normal endocervix (Fichorova et al. 2002). In our studies, TLR4 expression was positive, but not strong, in normal cervical epithelial cells, especially basal layer cells, and negative in normal cervical stroma, but the expression became gradually stronger in accordance with the histopathological grade from CINs to ICC. The pattern of expression of TLR4 in different compartments of the female reproductive tract may indicate a protective role of TLR4 against pathogens arriving from the female genital tract and in maintaining commensal organism homeostasis (Netea et al. 2004; Wira et al. 2005; O'Neill 2008). If cervical epithelial cells abnormally express TLR4, this could result in increased levels of circulating TNF, which could lead to the up-regulation of NF-kappaB anti-apoptotic factors, such as Bcl-Xl, cIAP1, and cIAP2, and an imbalance in homeostasis that potentially induces epithelial cell transformation and carcinogenic changes

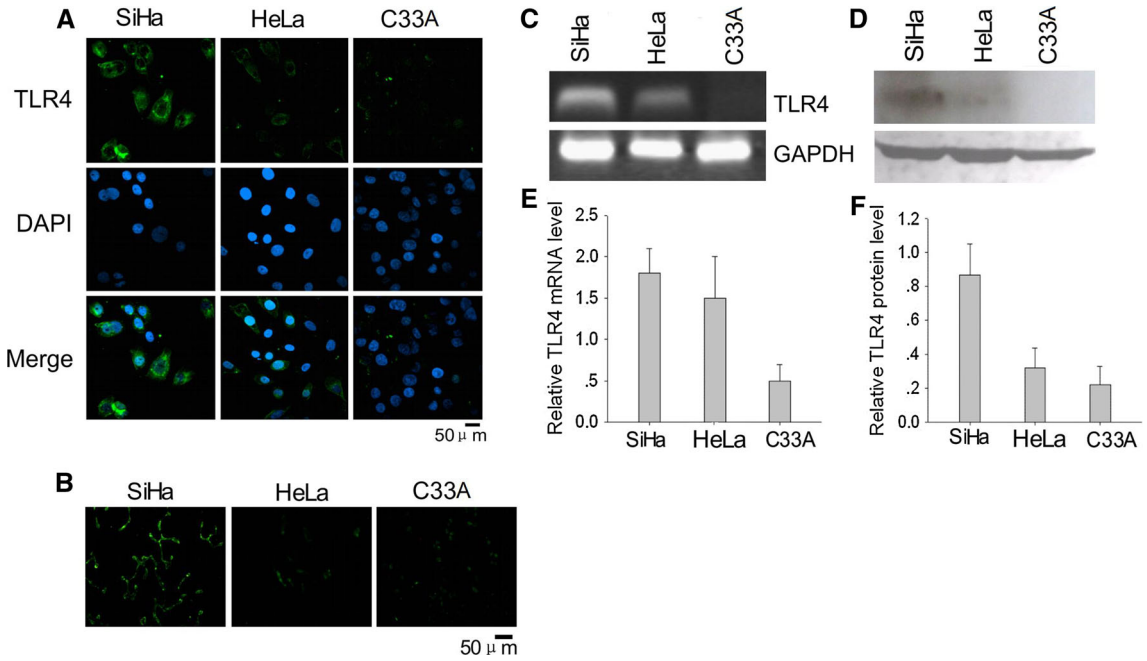

Fig. 2 Expression of TLR4 in SiHa, HeLa, and C33A cells. Images of the TLR4 protein were obtained by confocal laser scanning microscopy (**a**) and normal fluorescence microscopy (**b**). (**c**) Expression of TLR4 mRNA (160-bp PCR product) was detected by RT-PCR. GAPDH (238-bp PCR product) was co-amplified as the internal control. (**d**) Expression of the TLR4 protein (72 kD) was detected by Western blot analysis in total cell extracts. GAPDH (36 kD) was reprobed to confirm equal protein loading. Quantitative RT-PCR (**e**) and Western blot (**f**) data. Each *data point* in (**e**) and (**f**) represents the mean ± SD of three independent experiments. All results are normalized to those of GAPDH

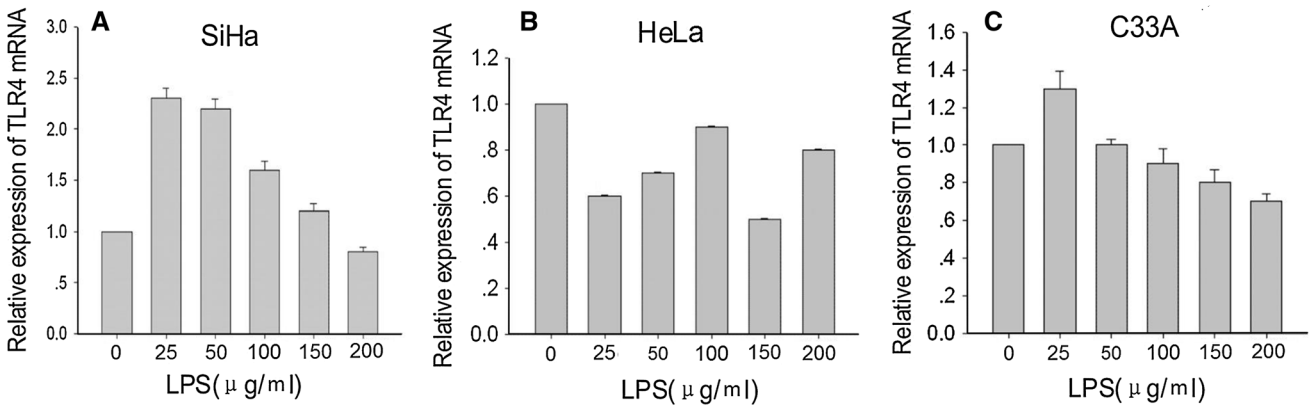

Fig. 3 Effects of LPS on the expression levels of TLR4 in SiHa (**a**), HeLa (**b**), and C33A (**c**) cells. All of the cells were exposed to 25, 50, 100, 150, or 200 μg/mL LPS for 24 h. The results are normalized to those of GAPDH mRNA and are expressed as the fold induction compared with the non-LPS treated group. The expression level of TLR4 mRNA for the 0 μg/mL LPS group is set at 1. The *bar graph* shows the results of three independent experiments

(Mantovani et al. 2008; Rakoff-Nahoum and Medzhitov 2009). While we have provided preliminary evidence that TLR4 expression in endocervical epithelial cells and vaginal bacterial conditions could be involved in the early events of cervical carcinogenesis, further studies are needed to determine the detailed mechanism.

Recent studies have revealed that chronic inflammation promotes carcinogenesis by multiple mechanisms (Mantovani et al. 2008). During the inflammatory response, TLRs, particularly TLR4, engage in shaping the tumor microenvironment, including cancer, inflammatory, immune, stromal, endothelial, and epithelial cells. Cancer cells depend on their microenvironment to provide signals for growth, antiapoptosis, angiogenesis, and metastasis, and the mutual interaction between transformed cells and the microenvironment controls tumor fate (Whiteside 2008). (Fukata et al. 2009) has shown that

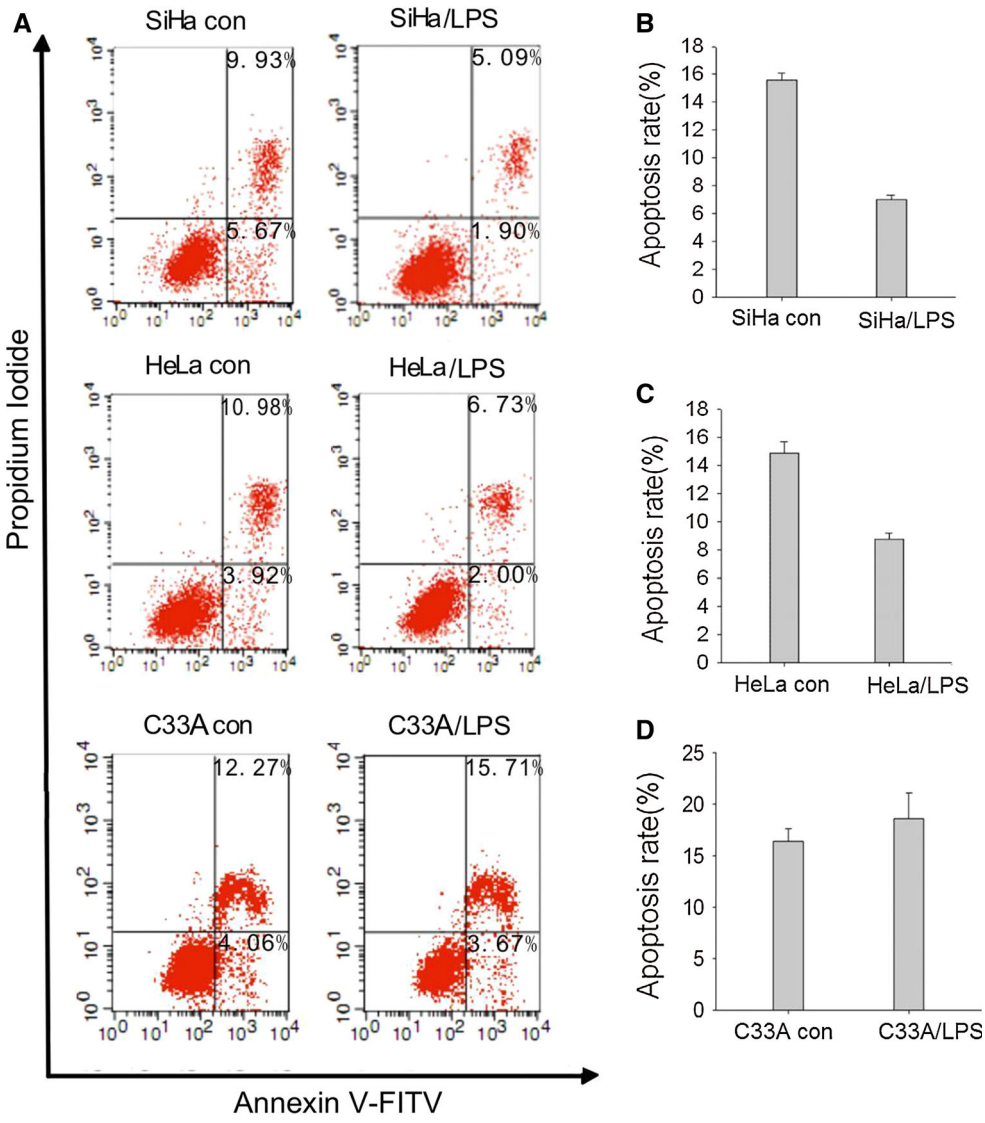

Fig. 4 The apoptosis rate and cell cycle distribution after 25 μg/mL LPS treatment for 24 h, using flow cytometric analysis. Untreated cells were analyzed as a control. (**a**) The flow cytometric results presented depict representative data. The panels are the quantitative data of the average apoptosis percentage in SiHa (**b**), HeLa (**c**), and C33A (**d**) cells with the indicated treatment. Each *data point* represents the mean ± SD of three replicates

Table 2 The percentages of the cell profiles by LPS treatment in Fig. 5

Cell cycle	SiHa		HeLa		C33A	
	Con (%)	/LPS* (%)	Con (%)	/LPS* (%)	Con (%)	/LPS* (%)
G0/G1	64.84 ± 3.12	67.39 ± 2.14	60.97 ± 3.16	49.44 ± 1.90	47.04 ± 1.73	45.97 ± 1.15
G2/M	6.65 ± 0.54	4.47 ± 0.42	6.86 ± 1.27	13.97 ± 0.93	11.63 ± 0.72	13.11 ± 1.04
S	28.52 ± 1.47	28.14 ± 1.95	32.17 ± 1.46	36.60 ± 2.07	41.32 ± 2.11	40.92 ± 2.39

Results of cell cycle analyses represent the mean ± SD of three independent experiments

*/LPS** LPS 25 μg/ml

innate immune signaling by TLR4 shapes the inflammatory microenvironment in colitis-associated tumors. Our study demonstrated that the expression levels of TLR4 gradually increased in accordance with the histopathological grade. These findings suggest that TLR4 may play an essential role in the initiation and development of cervical carcinoma and in shaping the tumor microenvironment.

We found that TLR4 expression was distinct in different HPV-status cervical cancer cells. It was functionally expressed positively in SiHa (HPV16+) and HeLa (HPV18+) cells, and absent in C33A cells (HPV−). When these cells were treated with LPS, SiHa and HeLa cells presented obvious apoptosis resistance, but C33A cells did not. Several studies have indicated that TLR4 is expressed

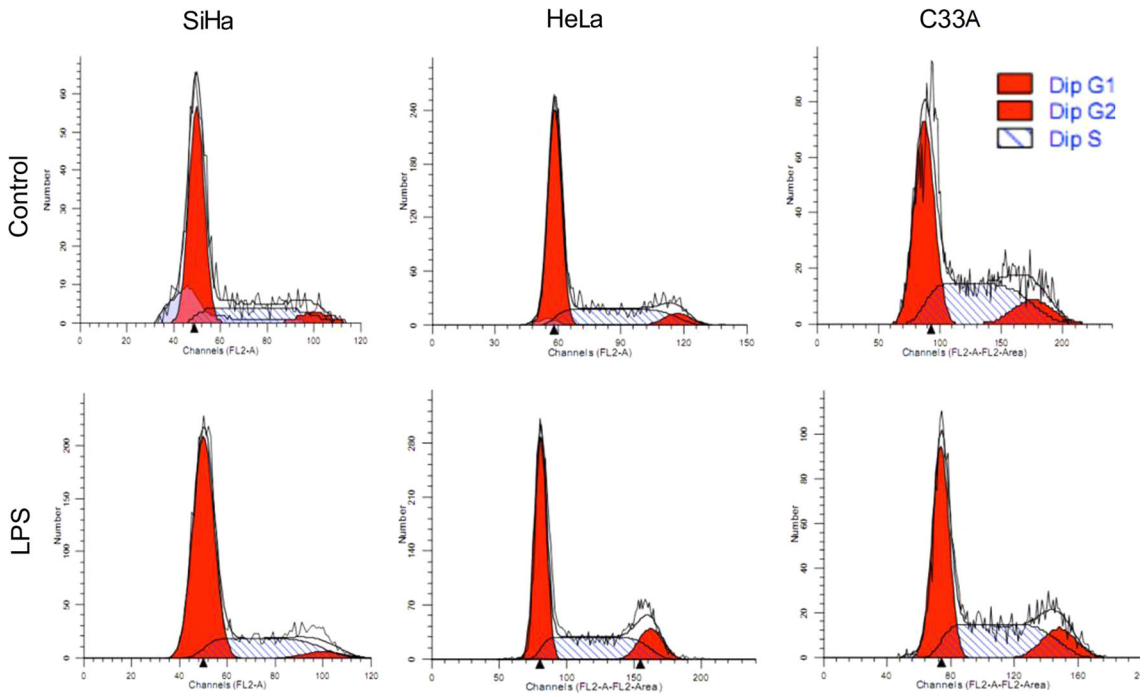

Fig. 5 Effect of 25 μg/ml LPS on the cell cycle distribution of SiHa, HeLa, and C33A cells by flow cytometric analysis. Flow cytometry was used to analyze changes in propidium iodide-stained cells. (**a**) The representative flow cytometric results show the cell cycle distribution of each group. The data indicate the mean percentage ± SD of three replicates

in some tumor cell lines and could promote tumor growth and cancer cell apoptosis resistance (Yu and Chen 2008; Kelly et al. 2006). Our results also showed similar apoptosis-resistant phenomena in SiHa and HeLa cells, which indicated that different HPV statuses could interfere with the expression and function of TLR4. This point can also explain the result indicating that TLR4 expression gradually increased with histopathological grade from CIN to ICC because more cases of HPV16 infection in ICC were found than in CINs and normal cervix. However, Yu et al. showed that TLR4 is down-regulated in high-risk, HPV16-positive cervical cancer cell lines (Yu et al. 2010). It is not clear why TLR4 is up-regulated in most high-risk, HPV16-positive cervical cancer cell lines but down-regulated in a few others. Moreover, (Werner et al. 2012) used 17 frozen cervical tissue biopsy samples and showed that TLR4 was undetectable in all but 1 biopsy sample. They showed high TLR4 mRNA expression levels in C33A cells and low levels in SiHa and HeLa cells. We cannot explain why different TLR4 expression levels were found in the same cells. For the biopsies, large numbers of cervical tissue biopsies were needed to determine whether TLR4 was detectable or not in cervical tissues. In addition, it is unclear how TLR4-induced apoptosis resistance occurs in these cancer cells. Our data in Fig. 5 and Table 2 showed no obvious change in the cell cycle distribution after LPS treatment of SiHa cells, which may indicate that apoptosis resistance was not related to cell cycle distribution.

Taken together, TLR4 was strongly expressed in cervical cancer tissues and HPV16-positive cancer cell lines. It induced apoptosis resistance and might participate in regulating carcinogenesis and tumor progression. However, additional studies are needed to elucidate the role of TLR4 as a promising therapeutic target and possible tumor marker for cervical cancer.

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Conflict of interest The authors have no conflicting financial interests.

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