

Original Article

# Induction of TLR5, IRAK1, and NF- $\kappa$ B expression by *Trichomonas vaginalis* in cervical cancer cell (HeLa) and normal human vaginal epithelial cell (HVECs) lines

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## Abstract

**Introduction:** Trichomoniasis is the most common non-viral sexually transmitted infection that increases the risk of cervical cancer. *Trichomonas vaginalis* (*T. vaginalis*) can regulate the pro-inflammatory cytokine production in the host cells. Toll-like receptors (TLRs) are a family of the pattern recognition receptors (PRRs) of mammalian cells, expressed in various host cells and have an important role in recognizing pathogens, and pro-inflammatory responses. The aim of the present study is to investigate the role of TLR5 in cervical cancer cells (HeLa) and human vaginal epithelial cells (HVECs) exposed to *T. vaginalis*.

**Methodology:** First, the cells and parasites were cultured in RPMI and trypticase yeast extract maltose (TYM), respectively. After adaption of parasite and epithelial cells by RPMI-TYM medium co-culture (9:1 vol/vol), HVECs and HeLa cells were stimulated with *T. vaginalis* trophozoites (24-hour incubation at 37 °C, 5% CO<sub>2</sub>). Following RNA extraction and cDNA synthesis, the gene expression levels of TLR5, IRAK1, and NF- $\kappa$ B were assessed using real-time PCR. Besides, the protein levels were measured using western blotting. All tests and controls were normalized using  $\beta$ -actin as a housekeeping control.

**Results:** Real-time PCR results showed an increased gene expression of TLR5, IRAK1, and NF- $\kappa$ B in *T. vaginalis* exposed HVECs and HeLa cells compared to the control group ( $p < 0.05$ ). Additionally, western blot analysis showed a statistically significant increase in TLR5, and NF- $\kappa$ B proteins in both groups after exposure to the parasite ( $p < 0.05$ ).

**Conclusions:** These findings provide insight into the host-parasite interaction, and the results indicated that *T. vaginalis* could stimulate TLR5 and activate related pathways.

**Key words:** Human vaginal epithelial cell; inflammation; *in vitro*; TLR5; trichomoniasis.

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## Introduction

*Trichomonas vaginalis*, a flagellated protozoan parasite, is responsible for the common sexually transmitted infection [1]. Trichomoniasis affects both female and male urogenital tracts, being mostly

asymptomatic, especially in males. In women, symptomatic cases often lead to vaginal discharge, odor, irritation, low birth weight, and increased risk of HIV and cervical cancer [2-4]. World health organization (WHO) estimates a global prevalence and

incidence of infection about 5.9 % and 156 million cases, respectively [5]. In the urogenital tract, mucus and epithelial cells act as a physical barrier and prevent microorganism invasion, such as *T. vaginalis*. This extracellular protozoan parasite infects individuals with the ability to degrade the mucus layer through proteinase [3]. Afterward, the parasite adheres to the host epithelial cells for colonization by binding to the host cell membrane galectins via its membrane lipophosphoglycan (LPG). *T. vaginalis* infection activates M2 macrophages, and induces Th2 responses. Th2 cytokines cause the activation, expansion, and differentiation of B lymphocytes into plasma cells that produce specific IgM, IgG and IgA antibodies in genital secretions and serum. Although humoral immunity plays an important role against extracellular pathogens, the parasite can escape from the host's humoral immunity. So, the cellular immune response appears to be more efficient in *T. vaginalis* infection [3,6].

On the other hand, stimulation of macrophages by *T. vaginalis* causes the differentiation of naive T cells to Th1 of acquired immunity. Afterward, Th1 cytokines including IFN- $\gamma$ , IL-2, and TNF- $\alpha$ , activate macrophages, cytotoxic T cells, and natural killer cells that can control and clear the parasite. However, the parasite produces exosome-like substances to escape the host's immune response. Exosome-like substances prevent the production of pro-inflammatory cytokines from macrophages and Th1 cells and subsequently cause the production of anti-inflammatory cytokines IL-10 and TGF- $\beta$  [6-8].

Indeed, macrophages act as an interface between the innate immune system and acquired immunity. The attachment of *T. vaginalis* to the epithelial cells stimulates TLR2, TLR4, and TLR9. Consequently, TLRs trigger intracellular cascades and inflammatory immune responses and causes the production and release of cytokines such as IL-6 and IL-8 [9,10].

TLRs are type I transmembrane proteins, as critical sensors of infection for the innate immune system, and are expressed in mammalian immune cells, such as dendritic cells and macrophages. TLRs are also expressed at low levels in non-immune cells, including fibroblasts, endothelial and epithelial cells. These receptors can identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). TLRs can be divided into two subgroups; cell membrane receptors (TLR1, TLR2, TLR4, TLR5, TLR6 in human cells, and TLR11 in mice) which sense external microbial components, including lipopeptides, flagellins, lipopolysaccharide, and intracellular receptors (TLR3, TLR7, TLR8, and

TLR9) which bind to nucleic acids derived from bacteria and viruses, and also self-nucleic acids [11,12].

Cell membrane TLRs have similar and common intracellular signaling pathways, although TLR5 directly recruits adaptor protein MyD88. TLR5 is expressed on the basolateral surface of epithelial cells and recognizes the conserved domain of the bacterial flagellin monomer [13]. Following TLR5 and flagellin attachment, MyD88 recruits interleukin 1 receptor-associated kinase-4 (IRAK-4) that phosphorylates IRAK-1 which in turn activates TNFR-associated factor 6 (TRAF6). Both leave the receptor complex and interact with transforming growth factor *beta*-activated kinase1 (TAK1) and two TAK1 binding proteins TAB1 and TAB2. Afterward, TAK1 becomes phosphorylated and activates the I- $\kappa$ B kinase (IKK) complex (IKK $\alpha$ , IKK  $\beta$ , and NEMO/IKK $\gamma$ ). Eventually, mitogen-activated protein kinase (MAPK), leads to the activation of NF- $\kappa$ B and c-Jun N-terminal kinases (JNK) signaling pathways [14].

As mentioned, TLR5 expressed on the host cells is known to involve in recognizing motile bacterial flagellin such as uropathogenic *Escherichia coli* [15]. According to the signaling cascade that is triggered and leads to the secretion of proinflammatory cytokines, bacterial products were screened for the ability to activate TLR5 and found that flagellin could activate NF- $\kappa$ B-mediated gene expression in TLR5-transfected cells [16]. On the other hand, scanning electron microscopic images showed that *T. vaginalis* adheres to human lung alveolar epithelial cells through its flagella and axostyle as well as cell membrane [17]. Nonetheless, the function of TLR5 against *T. vaginalis*, a flagellated protozoan parasite of the human reproductive tract, is still less clear. This study tries to understand the role of TLR5 signaling pathway in recognizing the *T. vaginalis* flagella and examined the expression of TLR5, IRAK1, and NF- $\kappa$ B via real-time PCR and western-blot methods in human normal vaginal epithelial cells (HVEC) and cervical cancer cells (HeLa) exposed to *T. vaginalis*.

## Methodology

### *Parasites and Cell Culture*

The *T. vaginalis* was kindly provided by Dr. Momeni (Karaj University, Iran). In the current study, the strain of *T. vaginalis* was isolated from the vaginal secretions of woman with acute vaginitis (H genotype, GenBank accession numbers: KP400513). *T. vaginalis* were cultured in Diamond's trypticase yeast extract-maltose medium (TYM) [18] with 10% bovine calf serum at 37 °C for 48 hours, and only the late-

logarithmic-phase trophozoites were used for the experiments.

Human cervical epithelial cells (HeLa cells) and human vaginal epithelial cells (HVECs) were obtained from the Immunology Research Center of Tabriz Medical Science University. HeLa cells and HVECs were cultured in RPMI 1640 culture medium (SIGMA), supplemented with 10% FBS, at 37 °C incubator and in the presence of 5% CO<sub>2</sub> up to reached confluence > 80% and logarithmic phase.

*Adaptation of T. vaginalis, HeLa cells, and HVECs with co-cultured medium*

T. vaginalis

RPMI was added to TYM medium in continuous culture using 10 serial passages up to obtaining RPMI-TYM medium (9:1vol/vol) as in the former studies carried out on the adaptation method [19]. Finally, *T. vaginalis* was cultured in the co-cultured RPMI-TYM medium and was used for the experiments.

HeLa cells and HVECs

The addition of TYM medium to RPMI medium was conducted by serial passages in a similar way to that of RPMI-TYM medium (9:1 vol/vol). Parasite/cell counting and viability assessment were performed using the manual trypan blue staining method [20]. Briefly, the cell or parasite suspension (approximately 1 × 10<sup>6</sup> cells or parasites/mL) was mixed with 0.4% trypan blue solution and let stay at room temperature for a few minutes. Then, cell survival was calculated using a hemocytometer slide (the number of viable cells divided by the total number of cells). Actually, viable cells will be colorless and have a clear cytoplasm whereas dead cells will have a blue cytoplasm (also about parasites).

*Co-culture of T. vaginalis with HeLa cells and HVECs*

After adaptation, HeLa cells and HVECs (5 × 10<sup>4</sup>) were seeded on a 24-well plate containing co-culture RPMI-TYM medium (9:1 vol/vol) at 37 °C and 5.0% CO<sub>2</sub> overnight. Then cells were exposed to three different ratios of *T. vaginalis* trophozoites (1X: 5×10<sup>4</sup>,

3X: 15×10<sup>4</sup>, 5X: 25×10<sup>4</sup>). Experiments were performed in triplicate for each test group.

*RNA extraction and cDNA synthesis*

After 24 hour exposure of the HeLa cells, and HVECs with *T. vaginalis*, the cells were washed with RPMI and adherent cells were harvested and lysed using 0.25% Trypsin/EDTA to a 25 mL-flask following incubation at 37 °C for 2-3 minutes. Total RNA was extracted with TRIzol® reagent (Gene All, Korea) and treated with DNase I. The RNA concentration was determined using NanoDrop (2000C, Thermo Scientific). Then cDNA was synthesized from total RNA using reverse transcriptase kit according to manufacturer directions (BioFact™ RT Kit, Cat.No. BR123-R10k).

*Real-Time PCR Measurement for TLR5, NF-κB and IRAK1 Gene Expression*

Real-time PCR was performed using 2X Real-Time PCR Master Mix (BioFACT™, Cat.No. DQ383-40 hours) with 20 ng of cDNA and specific primers (Table 1) in a light cycler instrument (Roche, Germany). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes; followed by 40 cycles of 95 °C for 20 seconds, annealing for 20 seconds (TLR5: 58 °C, NF-κB 60 °C, and IRAK1: 59 °C) (Table 1), 72 °C for 30 seconds and a final extension at 72 °C for 10 minutes. The expressions of mRNA levels of the analyzed genes were normalized to the amount of β-actin as a housekeeping gene.

*Western blotting assay for TLR5 and NF-κB*

*T. vaginalis* exposed HeLa cells and HVECs as mentioned above were lysed in a lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40) or 0.1% Triton X-100 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1mM sodium orthovanadate, 50 mM NaF and Protease inhibitors tablet (Roche)]. After centrifugation for 16,000 × g, 20 minutes at 4 °C, the supernatant was collected. Protein concentrations were measured using the Bradford protein-quantification assay [21]. Then, 20

**Table 1.** TLR5, NF-κβ and IRAK1 primer sequences and related annealing temperatures.

Gene	Primer sequences	Annealing
TLR5	F:5'CTTCCTGCTCCTTTGATGGC3' R:5'TCCTGATATAGTTGAAGCTCAGC3'	58°C
NF-κB	F:5' GGCTACACAGGACCAGGGAC3' R:5' AGAGCTCAGCCTCATAGAAG3'	60°C
IRAK1	F: 5'CGTACACCAGGCAGTAGAAG3' R: 5'TGAAGAGGCTGAAGGAGAAC3'	59°C
β-actin	F: 5'-CACGATGGAGGGCCGGACTCATC-3' R: 5'-TAAAGACCTCTATGCCAACACAGT-3'	56°C

µg of each sample was taken and an equal volume of 2x Laemmli sample buffer was added. Each cell lysate was boiled in the sample buffer at 95 °C for 5 minutes and centrifuged at 16,000 g for 1 minute. Then, 20 µg of lysate (protein suspension) from each sample was loaded on 15% SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane. PVDF membrane was blocked in blocking buffer (Tris buffered saline with Tween (TBST) containing 3 % bovine serum albumin (BSA) at room temperature for 1 hour. The membrane was incubated with primary antibodies (β-actin sc-69879, Santa Cruz Biotechnology, TLR5 sc-517439, Santa Cruz Biotechnology, and anti-NF-κB p65 antibody ab16502, Abcam) overnight at 4 °C. The blot was rinsed 3–5 times for 5 minutes with TBST and was finally incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Anti Rabbit, SC-2004, Santa Cruz) solution for 1 hour at room temperature.

The membrane was developed with chemiluminescent substrate according to the manufacturer’s recommendation and chemiluminescent signals were captured using a CCD camera-based imager. We used ImageJ analysis software to read the band intensity of the target proteins. Densitometry analysis of the TLR5 and NF-κB protein levels were expressed as relative fold changes after normalization to the level of β-actin as an internal control.

**Statistical evaluation**

All experiments were performed twice using three replicates. One Way ANOVA test and Paired t-test were used for statistical analysis, and a *p* value below 0.05 was considered statistically significant. Graph Pad Prism 6 software was used for statistical evaluation.

**Ethical statement**

This study was approved by the Ethics Committee of Tabriz Medical Science University (Protocol no. IR.TBZMED.VCR.REC.1398.078).

**Results**

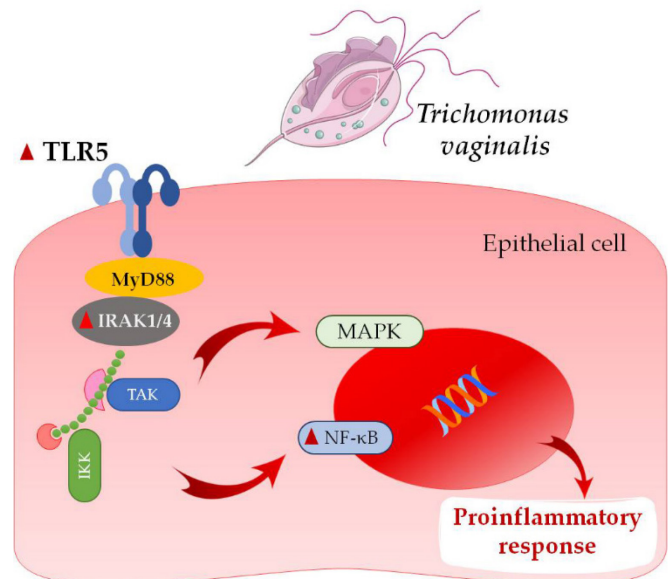
Gene and protein expression of TLR5, IRAK1, and NF-κB were investigated in HeLa cells and HVECs stimulated with *T. vaginalis* (Figure 1).

The analysis of Real-time PCR results revealed that the gene expression of TLR5, IRAK1, and NF-κB increased significantly *vs.* controls in HeLa cells stimulated with tree different ratios 1X, 3X and 5X of *T. vaginalis* for 24 h approximately as follow: TLR5, 700, 900, and 1550 fold change; IRAK1, 1.6, 2.2 and

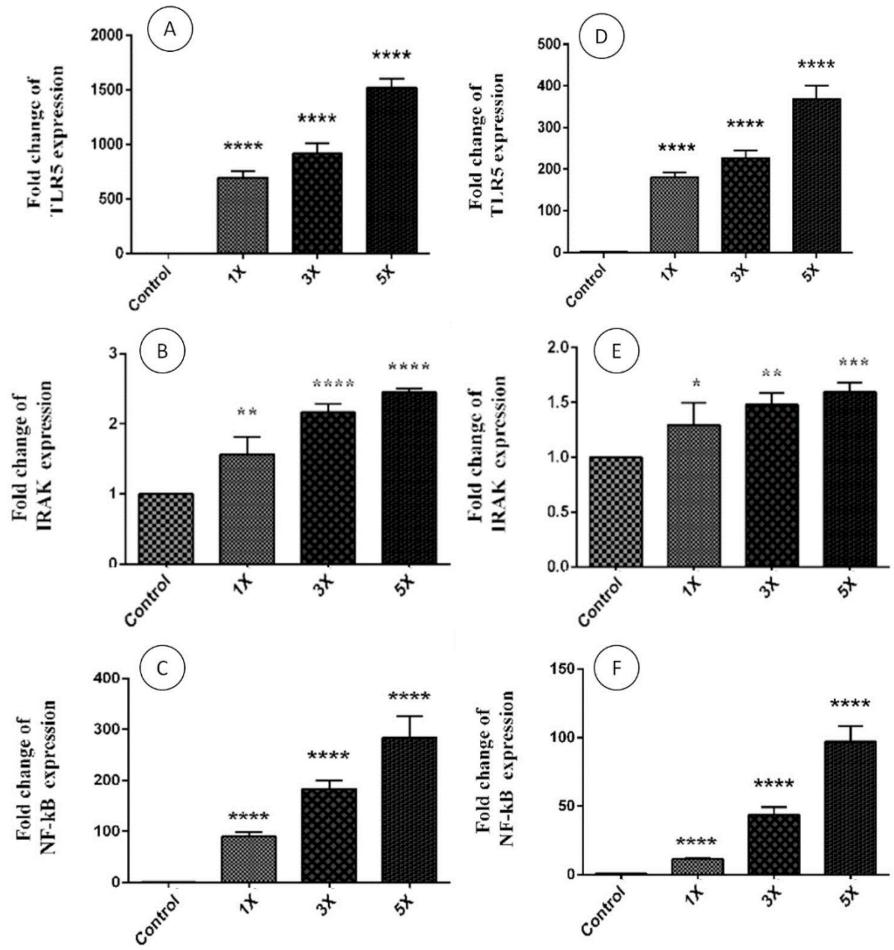
2.5 fold change; NF-κB, 90, 180 and 280 fold change, respectively (Figure 2 A, B, C).

Also, *T. vaginalis* was able to induce the expression of target genes significantly in HVECs compared to controls after 24 hour incubation at 1X, 3X, and 5X ratios approximately as follows: TLR5, 180, 220 and 360 fold change; IRAK1, 1.3, 1.5, and 1.6 fold change (Figure 2 D, E, F). Gene expression of TLR5, IRAK1, and NF-κB in HeLa cells and HVECs were boosted by increasing the parasite ratio and generally were higher in HeLa cells than HVECs (Figure 2). β-actin gene is used as a housekeeping gene to normalize tests. In addition to assessing gene expression, TLR5, and NF-κB were examined at the protein level in *T. vaginalis* exposed HeLa cells and HVECs by western blotting. In the case of IRAK1, due to low fold-change in gene expression, this gene was not examined by Western blotting. In HeLa cells stimulated with *T. vaginalis* for 24 hours, TLR5, and NF-κB proteins increased significantly compared to control (Figure 3). In HVECs incubated with *T. vaginalis* for 24h, both TLR5, and NF-κB proteins increased significantly in 5:1 ratio of parasite and epithelial cells (Figure 4). TLR5, and NF-κB proteins were higher in HeLa cells than HVECs. All tests normalized by β-actin protein as an internal control.

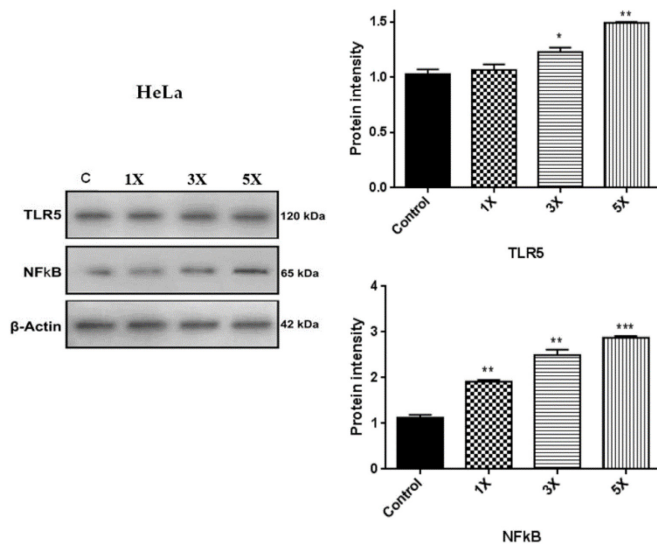
**Figure 1.** Epithelial cells exposed to *T. vaginalis* exhibited increased (Δ) TLR5, IRAK1 and NF-κB gene expression. Concomitantly, NF-κB and TLR5 protein expression were also increased. This suggests that TLR5 is involved in recognizing this flagellated protozoan parasite (flagellin) and contributes to a pro-inflammatory response via IKK –related pathways.



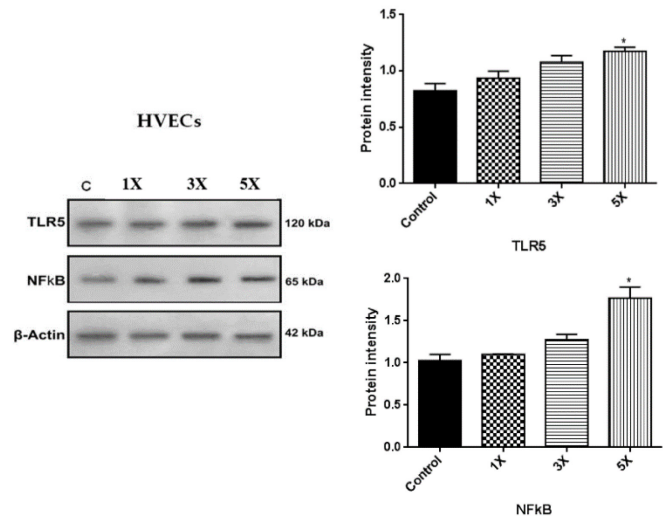
**Figure 2.** Gene expression of TLR5, IRAK1 and NF-κB in both HeLa cells (A, B, C) and HVECs (D, E, F) stimulated with *T. vaginalis* measured by Real- Time PCR. Results show significant increase compared with control (ns  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs. control).



**Figure 3.** Western blotting analysis of HeLa cells: Image and plot of TLR5 and NF-κB proteins compared with control. β-actin protein was used as an internal control protein to normalized target proteins. The 1:1 (1X), 3:1 (3X), and 5:1 (5X) ratio of parasite and epithelial cells were used for western blot analysis.



**Figure 4.** Western blotting analysis of HVEC cells: Image and plot of TLR5 and NF-κB proteins compared with control. β-actin protein was used as an internal control protein to normalized target proteins. The 1:1 (1X), 3:1 (3X), and 5:1 (5X) ratio of parasite and epithelial cells were used for western blot analysis.



## Discussion

Trichomoniasis is associated with an increased risk for other sexually transmitted diseases. There is also a relationship between *T. vaginalis* infection and cervical cancer [4,22]. Multiple exposures, infection with a high number of parasites, and re-infection may increase the inflammation and potentially lead to tissue damage. Infectious agents such as parasites can activate inflammation and trigger inflammatory signaling pathways [23,24]. Additionally, chronic inflammation increases the risk of several cancers and is considered to be one of the characteristics of tumor initiation and facilitates tumor progression [24-26]. Therefore, *T. vaginalis* has been introduced as one of the predisposing factors for cervical cancer. The current study investigates the expression of 1:1, 3:1, 5:1 ratios of parasites: cells by real-time PCR and western blot analysis. The results showed that exposing HeLa (cancer cell) and HVECs (normal cell) to *T. vaginalis* led to the stimulation of TLR5 and activation of its downstream signaling pathway. Real-time PCR results indicated a significant increase in TLR5, IRAK1, and NF- $\kappa$ B mRNAs in both cell lines. Likewise, western blotting method confirmed that the level of TLR5, and NF- $\kappa$ B proteins increased significantly in cells exposed to the parasite.

TLRs, a class of mammalian PRRs, are expressed in host cells and act as important immunologic biosensors for pathogens components of bacteria, parasites, viruses, and fungi, and induce immune responses to limit or eradicate pathogens [27,28]. Previous studies have shown the role of TLR2, TLR4, and TLR9 in recognizing protozoan parasites such as *Leishmania spp.*, *Trypanosoma cruzi*, *Plasmodium falciparum* and *T. vaginalis* [14,29]. In the human female reproductive tract, vaginal and cervical epithelial cells express TLR1, 2, 3, 4, 5, 6, and 9 [30]. According to previous studies, *T. vaginalis* regulates the production of pro-inflammatory cytokines through p38, ERK, and NF- $\kappa$ Bp65 signaling pathways via stimulating TLR2 in mouse macrophages [31]. It is known that *T. vaginalis* could up-regulate TLR2, 4, and 9 gene expression via the p38 MAPK signaling pathway in HeLa cells [32].

Among TLR family, TLR5 is the only TLR involved in sensing bacteria flagellin of motile bacteria, such as *Enterobacteriaceae*, and *Helicobacter pylori* with stimulates innate and adaptive immune responses [33-35]. On the other hand, *T. vaginalis* is a protozoan parasite of the human urogenital tract which has flagella as motion means, although there are differences between prokaryotes (bacteria) and eukaryotes

(parasites) flagella; for example, bacterial flagella are formed from flagellin protein while parasite flagella subunits are formed from tubulin [36]. To date, the function of TLR5 in parasitic infections has remained less clear [15,35] and to the best of the authors' knowledge, this is the first study to examine the TLR5 role in *T. vaginalis* exposed epithelial cells. It was stated that *T. vaginalis* flagella may activate TLR5 in similar mechanisms of bacterial flagella [37]. In a study that explored the role of human TLR5 in the recognition of *Toxoplasma gondii* profilin, it was found that human peripheral blood monocytes and human embryonic kidney (HEK) 293 cell lines were activated by *T. gondii* profilin in a TLR5-sensitive manner [38]. On the contrary, a study by Tombacz *et al.* showed that *T. gondii* profilin does not stimulate an innate immune response through bovine or human TLR5 [39]. *Eimeria tenella*, a protozoan parasite, causes coccidiosis in chickens. Studying the roles of TLRs in chicken experimentally infected with *Eimeria tenella* shows that TLR5 gene expression together with TLR11A, TLR4, TLR7, and TLR21 significantly increased in cecum intraepithelial lymphocytes [40]. Also during *T. spiralis* infection, a worm parasite, in the small intestine and muscle tissue, it was demonstrated that the expression of TLRs genes significantly increased at 4 weeks post-infection in the muscle [41].

The results of the present study show, TLR5, IRAK1, and NF- $\kappa$ B mRNAs and TLR5, and NF- $\kappa$ B protein levels increased in HeLa cancer cells more remarkably than in HVECs normal cells. This could be due to the cancerous nature of HeLa cells or naturally high expression of mRNA compared to HVECs normal cells. Also, high parasite load causes more increase in gene expression and protein levels. Previous studies have shown that parasite adhesion to host cells and its subsequent effects were dose-dependent [42]. Also, Chang *et al.*, showed that expression of TLR2, TLR4, and TLR9 genes in *T. vaginalis* stimulates HeLa cells p38 MAPK signaling dependence manner [32].

## Conclusions

Based on the findings of the current study, TLR5 is probably involved in recognizing *T. vaginalis*, a flagellated protozoan parasite, that can activate TLR5-dependent pathway in female genital tract epithelial cells and up-regulate gene expression, like flagellated bacteria. On the other hand, upregulation of the TLR5 may be a consequence of the pro-inflammatory response. Interestingly, mRNA expression and protein level of proinflammatory cytokines in HeLa cancer cells were higher than that of HVECs normal cells. This

can indicate the fact that *T. vaginalis* can play a role in the induction and progression of cervical cancer by increasing inflammatory responses and stimulating innate immunity.

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### Ethics approval and consent to participate

All experiments were approved by the local Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (No. IR.TBZMED.VCR.REC.1398.078).

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