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Running title

E.coli contamination in endometriosis

Correspondence

***Escherichia coli* contamination of menstrual blood and effect of bacterial endotoxin on endometriosis**

Khaleque Newaz Khan^{1*}, MD, PhD; Michio Kitajima¹, MD; Koichi Hiraki¹, MD; Naohiro Yamaguchi², MD; Shigeru Katamine², MD; Toshifumi Matsuyama³, MD; Masahiro Nakashima⁴, MD; Akira Fujishita⁵, MD; Tadayuki Ishimaru⁶, MD; Hideaki Masuzaki¹, MD

¹Department of Obstetrics and Gynecology, ²Department of Molecular Microbiology and Immunology, ³Department of Cytokine Signaling, Nagasaki University Graduate School of Biomedical Sciences; ⁴Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki; ⁵Nagasaki Saiseikai Hospital, Nagasaki; ⁶Sasebo Chuo Hospital, Nagasaki, Japan

Conflict of interest: There is no conflict of interest related to this study

Financial support: This work was supported by Grants-in-Aid for Scientific Research (Grant No. 16591671 and 18591837) from the Ministry of Education, Sports, Culture, Science and Technology of Japan (to KN Khan).

* **Address for correspondence and reprint request:**

Khaleque Newaz Khan, MD, PhD

Department of Obstetrics and Gynecology

Graduate School of Biomedical Sciences, Nagasaki University

1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Tel: +81-95-819-7363

Fax: +81-95-819-7365

Email: nemokhan@nagasaki-u.ac.jp

Capsule

Higher contamination of *Escherichia coli* (*E.coli*) in menstrual blood and endotoxin (LPS) levels in menstrual fluid and peritoneal fluid may promote Toll-like receptor 4 (TLR4)-mediated growth of endometriosis.

Abstract

To test the hypothesis that bacterial contamination of menstrual blood could be a local biological event in the development of endometriosis, menstrual blood was cultured and bacterial endotoxin (lipopolysaccharide, LPS) was measured in menstrual blood and peritoneal fluid (PF). Our results suggest that comparing to control women, higher colony formation of *Escherichia coli* (*E.coli*) in menstrual blood and endotoxin levels in menstrual fluid and PF in women with endometriosis may promote Toll-like receptor 4 (TLR4)- mediated growth of endometriosis.

Key words: endometriosis, menstrual blood, *Escherichia coli*, peritoneal fluid, macrophages, TLR4, cell growth

Endometriosis is a chronic inflammatory disease involving secondary inflammatory mediators (1-4). Primary inflammatory mediators, e.g., bacterial endotoxin or lipopolysaccharide (LPS), triggers the secretion of various secondary inflammatory mediators, such as cytokines, chemokines and growth factors, by mature/activated macrophages in the pelvis (5). Bailey et al. (6) reported that endometriosis was associated with an altered profile of intestinal microflora in rhesus monkeys, and that endometriosis was linked to higher concentrations of Gram-negative bacteria. Based on these studies, we speculated that as carriers of LPS, different Gram-negative bacteria such as *Escherichia coli* (*E.coli*) residing in the vagina could be involved in human endometriosis.

The human lower genital tract is constantly exposed to various microorganisms (7-9), which could infect the upper genital tract through direct migration. We tested the hypothesis that as normal microbial flora in the vagina, some Gram-negative bacteria infect the uterine wall after ascending migration from the vagina to contaminate menstrual blood, consequently resulting in accumulation of endotoxin in the menstrual/peritoneal fluid and initiation of pelvic inflammation. The aim of the present

study was to assess the stimulatory effect of *E. coli*-derived LPS on the secretion of various macromolecules by macrophages known to be involved in endometriosis (3, 4). We also assessed the growth promoting effect of LPS on endometrial cells and the role of Toll-like receptor 4 (TLR4), a receptor recognizing LPS (7, 10), in the secretion of macromolecules by macrophages and growth of endometrial cells.

With informed consent, we recruited women who had regular ovulatory cycles. The Institutional Review Board Of Nagasaki University Faculty of Medicine approved this project. Peritoneal fluid (PF) was collected from 58 women with endometriosis (age, 20-42 years) and 28 control women (age, 18-32 years) without endometriosis. All subjects underwent a laparoscopy to confirm presence or absence of endometriosis.

Under strict asepsis, menstrual blood was collected from 20 women with endometriosis and 15 control women on days 1-3 of the menstrual cycle using the method described previously (11). Each sample was cultured in triplicate in a plate with eosin-methylene blue (EMB) agar (Difco Laboratories, Detroit, MI), which is rich in nutrients and allows the growth of *E. coli* (6). The limulus amoebocyte lysate test

(Endotoxin-Single Test; Wako-Jun-Yaku Co., Tokyo, Japan) was used to analyze bacterial endotoxin in menstrual and PF samples (11).

Macrophages from PF and epithelial/stromal cells were collected from the biopsy specimens of the eutopic/ectopic endometria of women with or without endometriosis, as described previously (12-17). After the first passage, macrophages (10^5 per well in Roswell Park Memorial Institute medium) were serum-starved for 24 hours and cultured for another 24 hours with variable doses of LPS derived from *E. coli* (serotype 0111:B4; Sigma, St. Louis, MO). Macrophages were also pre-incubated in the presence of polymyxin B (Sigma) (1 $\mu\text{g}/\text{mL}$), a potent LPS inhibitor, or with anti-TLR4 neutralizing antibody (10 $\mu\text{g}/\text{mL}$) (HTA-125, HyCult Biotechnology), for 20 min and then stimulated with 10 ng/mL of LPS for another 24 hr to examine changes in the secretion of macromolecules.

The concentrations of hepatocyte growth factor (HGF), vascular endothelial cell growth factor (VEGF), interleukin (IL)-6, and tumor necrosis factor alpha ($\text{TNF}\alpha$) in culture media were measured in duplicate by using an ELISA kit (Quantikine, R&D System, Minneapolis, MN). Immunohistochemical staining for TLR4 was performed by

using the HTA-125 antibody (dilution, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (12, 18).

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Tokyo, Japan). RNA (1 μ g) was added to reverse the transcription reaction (RT-PCR), and cDNA (1 μ L) was subjected to real-time qPCR using an ABI 7900HT system (Applied Biosystems, Warrington, U.K). All primers and probes were designed as described previously (12, 14, 19). The gene expression levels of TLR4, HGF, and its receptor c-Met were calculated and normalized by dividing the corresponding values of β -actin.

The mitogenic activity of cells was determined by 5-Bromo-2-deoxyuridine (BrdU) proliferation ELISA (Amersham Pharmacia, Tokyo, Japan). Data are expressed as mean \pm SEM or SD, or median and inter-quartile range (IQR) values for log transformed colony forming units (CFU) of *E. coli* per milliliter of menstrual blood. Results were analyzed by analysis of variance. Mann-Whitney U-test and Student's t-test were used to analyze differences between groups and Kruskal-Wallis test was used to analyze differences among groups. A *p* value of <0.05 was considered to be statistically significant.

The CFU/mL of *E. coli* in menstrual blood was significantly higher in women with endometriosis (median, Log₁₀, 4.5 CFU/mL; IQR, 1.4-7.2) than in control women (median, 1.2, IQR 0.8-1.9; p<0.01) (Figure 1A). Furthermore, the CFU/mL of *E. coli* was significantly higher in the menstrual blood of women with pelvic endometriosis with coexisting blood-filled opaque red lesions (20), than in that of women with only ovarian endometrioma without any coexisting red lesions (p<0.01, Figure 1B).

The endotoxin levels in menstrual/peritoneal fluid samples from women with endometriosis were significantly higher than in control women (menstrual, 285.5±64.5 pg/mL vs. 114.9±17.0 pg/mL, p<0.01; peritoneal, 71.5±9.2 pg/mL vs. 43.3±9.8 pg/mL, p<0.001, Figure 1C). The maximum level of endotoxin in peritoneal fluid was noted during the menstrual phase when compared with other phases of the menstrual cycle (Figure 1D).

The concentrations of HGF, VEGF, IL-6 and TNF α were significantly higher in the culture media of *E. coli* LPS-treated than -non-treated macrophages (p<0.05, for each, Figure 1E) and in women with endometriosis than that in control women (data not shown). This effect of LPS was abrogated after pre-treatment of cells with antibody

against TLR4 ($p < 0.05$, for HGF, IL-6, TNF α , $p < 0.01$ for VEGF, Figure 1E). This was confirmed at the gene level for HGF and its receptor c-Met (Supplemental Fig. 1). The cellular specificity of LPS was confirmed by similar significant decreases in the levels of all these macromolecules after pre-treatment of macrophages with polymyxin B and anti-TLR4 antibody ($p < 0.05$ for VEGF, IL-6, TNF α , $p = 0.08$ for HGF, Figure 1E).

The protein and mRNA expressions of TLR4 were detected in isolated stromal/epithelial cells as well as in intact gland/stromal cells derived from the eutopic/ectopic endometria of women with and without endometriosis (Supplemental Fig. 2). RT-qPCR analysis showed a substantial increase of TLR4 gene expression in both gland/stromal cells in the secretory phase compared with the proliferative phase of the menstrual cycle, with a higher expression in women with endometriosis than in those without endometriosis (Supplemental Table 1). The TLR4 gene and protein expression levels in macrophages are described elsewhere (3, 21).

LPS induced dose-dependent BrdU incorporation into epithelial/stromal cells derived from the eutopic and ectopic endometria of women with endometriosis (Figure 1F). The cell proliferation effect of LPS was remarkably higher in endometriosis women

than in control women (data not shown) and was significantly suppressed after pretreatment of cells with anti-TLR4 antibody ($p < 0.05$ for both epithelial cells and stromal cells, Figure 1F).

Our study demonstrated for the first time that menstrual blood of women with endometriosis is more contaminated with *E. coli* than that of control women and corresponds to higher levels of endotoxin in the menstrual fluid and consequently in the peritoneal fluid due to reflux of menstrual blood into the pelvis. This may promote TLR4-mediated growth of endometriosis as evidenced by the increased endometrial cell growth in response to LPS and abrogation of these LPS-mediated effects by anti-TLR4 antibody. Because the role of infection in endometriosis was elusive until now, here we propose a novel concept of “bacterial contamination hypothesis” for the development of endometriosis via LPS/TLR4-mediated engagement of innate immune response.

We reported previously a significant increase in infiltration of macrophages in eutopic/ectopic endometria and in the peritoneal fluid of women with endometriosis compared with control women (18, 22). In the study, we demonstrated that in addition to direct inflammatory reaction, *E. coli*-derived endotoxin induced macrophage- and

TLR4-mediated higher pro-inflammatory reactions in the pelvis of women with endometriosis compared with control women. In fact, we could not completely exclude misclassification error in the control group due to subtle lesions or detection failure by the surgeon.

Two mechanisms could explain the residual accumulation of bacterial endotoxin in the pelvis. (1) translocation of *E. coli* or endotoxin from the gut through enterocytes and their entry into the pelvis (23, 24); or (2) contamination of menstrual blood by *E. coli* after its migration from the vagina to the uterine cavity and its replication by some unknown mechanism. We speculate that contamination of menstrual blood with *E. coli* could be a constant source of bacterial endotoxin in the peritoneal cavity that may promote TLR4-mediated growth of endometriosis. We postulate that migrating *E. coli* is the initial contaminant of menstrual blood, which then induces pelvic inflammation and finally TLR4-mediated growth of endometriosis.

Our results carry some biological and clinical implications in endometriosis.

(1) In addition to the traditional transplantation and celomic metaplasia theory, we propose a novel “bacterial contamination” hypothesis for the development or maintained

growth of endometriosis via LPS/TLR4 pathway; and (2) targeting TLR4 could be a potential therapeutic strategy to reduce pelvic inflammation, symptoms and growth of endometriosis. We believe that the “bacterial contamination” concept could facilitate further research into the physiopathology of endometriosis.

Acknowledgments: We thank Dr. Shingo Moriyama, Miss Kazumi Hayashida and Miss Kyoko Ishida, Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, for their excellent technical assistance.

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Figure Legends

Figure 1. (A) Colony forming units (CFU) of *Escherichia coli* per milliliter of menstrual blood, which was collected from women with endometriosis (red box) and control women (blue box) and was expressed as log transformed CFU/mL. A significantly higher colony formation of *E. coli* was observed in the menstrual blood of women with endometriosis than that of control women ($p < 0.01$). (B) Women with pelvic endometriosis who had coexisting red peritoneal lesions (red) showed significantly ($p < 0.01$) higher colony formation of *E. coli* in their menstrual blood than women with ovarian endometrioma (green) who had no coexisting red peritoneal lesions. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (C, D) Endotoxin levels in the peritoneal fluid (PF) and menstrual fluid (MF) of women with and without endometriosis are shown. The endotoxin level was significantly higher in both MF ($p < 0.01$) and PF (< 0.001) collected from women with endometriosis (solid bars) than from control women (open bars) (C). The highest

endotoxin level was found during the menstrual phase and a modest level in the proliferative phase or the secretory phase **(D)**. The level of endotoxin was significantly higher in the PF derived from women with endometriosis than that from the control women **(D)**. Results are expressed as mean \pm SEM.

(E) The levels of HGF, VEGF, IL-6 and TNF α in culture media of macrophages derived from the PF of women with endometriosis and in response to LPS treatment and non-treatment (control) are shown. The levels of these macromolecules were significantly higher in the treated group than in the non-treated group (open bars) and their levels were significantly decreased when macrophages were pretreated with either anti-TLR4 antibody (solid bars) or polymyxin B, a potent LPS antagonist (hatched bars). Results are presented as mean \pm SEM of three different experiments. **(F)** BrdU incorporation into glandular epithelial cells (open bars) and stromal cells (solid bars) of eutopic (left) and ectopic endometria (right) derived from women with endometriosis is expressed by the percentage of control (non-treated) cells. Both epithelial cells and stromal cells proliferated dose-dependently in response to the indicated doses of LPS and this augmented cell proliferation was significantly suppressed after pretreatment of cells

with anti-TLR4 antibody. The results are presented as mean \pm SEM of three experiments.

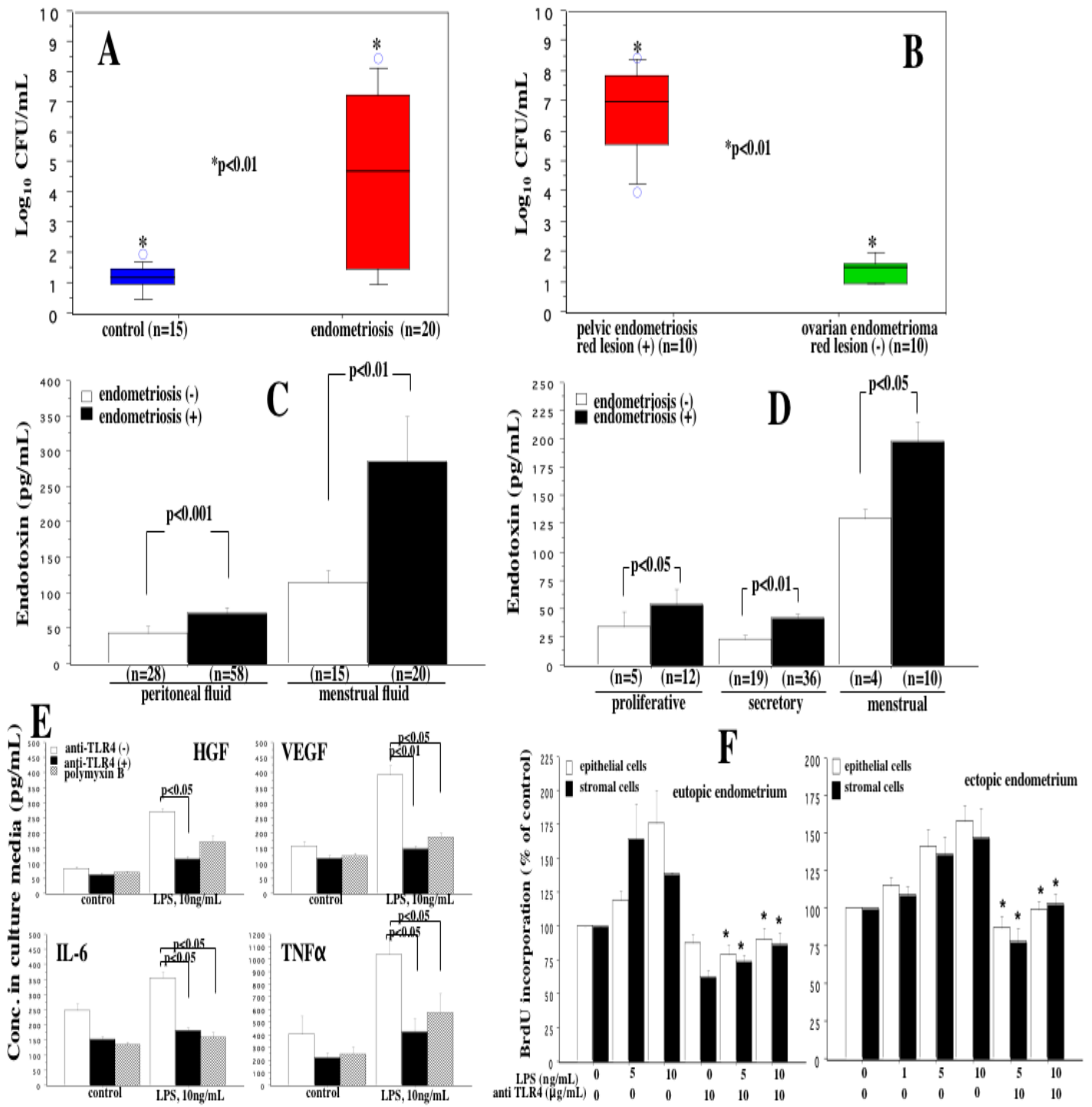
* $p < 0.05$, compared with cells not treated with anti-TLR4 antibody.

Supplemental Figure 1. (A) Effects of LPS on the mRNA expression levels of HGF and its receptor, c-Met in macrophages derived from the peritoneal fluid (PF) of women with and without endometriosis. The gene expression levels of HGF (505 bp) and c-Met (536 bp) were dose-dependently increased in response to LPS and this effect was higher in macrophages derived from the PF of women with endometriosis compared with that from women without endometriosis. **(B, C)** The LPS-stimulated augmented expression levels of HGF and c-Met gene were significantly abrogated when macrophages were pretreated with anti-TLR4 antibody (* $p < 0.05$ vs. without anti-TLR4 antibody) **(B, C)**.

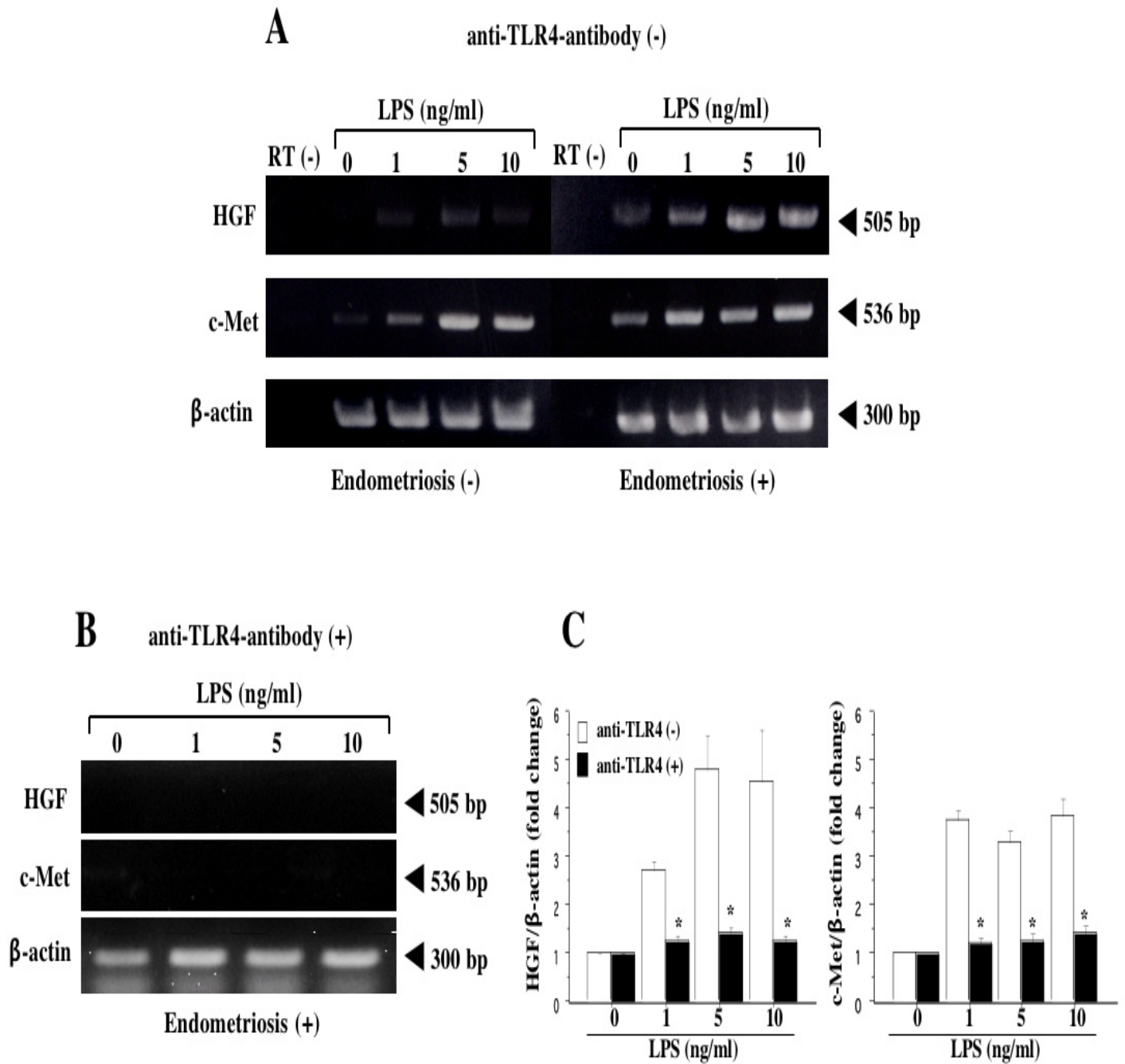
Supplemental Figure 2. Immunoreactivity and gene expression levels of Toll-like receptor 4 (TLR4) are shown in the eutopic and ectopic endometrial cells **(A, B, top)**. The immunoexpression of TLR4 was found in cytokeratin-positive epithelial cells,

vimentin-positive stromal cells and the eutopic endometria of women with and without endometriosis (**A**, **B**, left and right upper panels). (**C**) The mRNA expression levels of TLR4 (406 bp) were detected in the eutopic epithelial and stromal cells during the proliferative phase (P) and the secretory phase (S) of the menstrual cycle. (**D**) A similar pattern of TLR4 gene expression was also observed in the ectopic epithelial cells (E) and stromal cells (S) derived from women with pelvic endometriosis and during the proliferative phase and the secretory phase of the menstrual cycle.

Figure 1



Supplemental Figure 1



Supplemental Figure 2

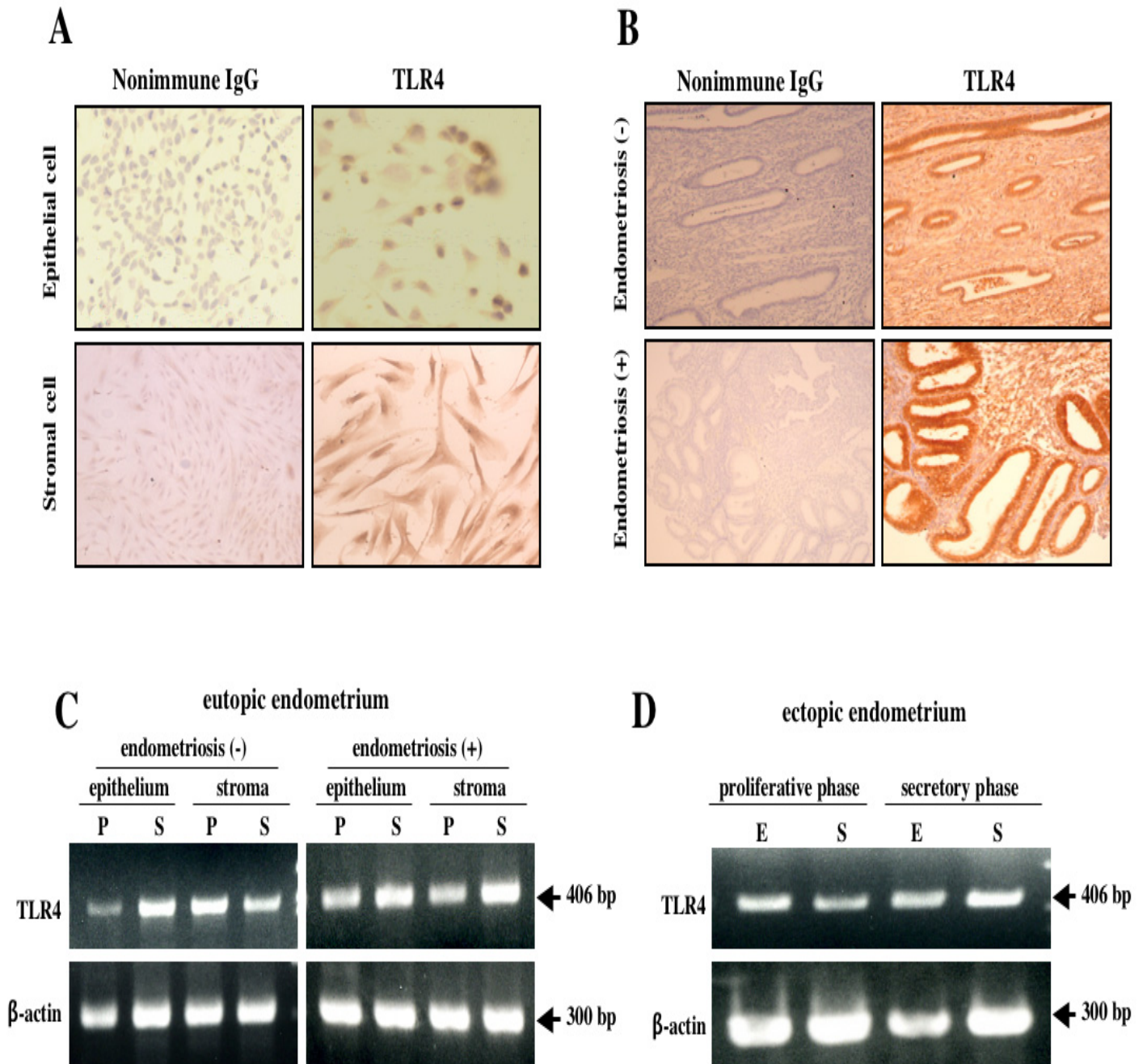


Table 1. Relative gene levels of TLR4 in the eutopic and ectopic endometria according to menstrual cycle.

	eutopic endometrium				ectopic endometrium			
	epithelium		stroma		epithelium		stroma	
	proliferative	secretory	proliferative	secretory	proliferative	secretory	proliferative	secretory
endometriosis (-)	0.68 ± 0.15	0.97 ± 0.11	1.05 ± 0.12	1.12 ± 0.22				
endometriosis (+)	0.88 ± 0.12	1.12 ± 0.15	1.15 ± 0.17	1.23 ± 0.13	1.28 ± 0.12	1.32 ± 0.15	1.14 ± 0.16	1.42 ± 0.22

Non-treated eutopic and ectopic endometrial cells were cultured derived from the proliferative phase and the secretory phase of women with and without endometriosis. The expression of TLR4 mRNA was determined using real-time quantitative PCR and was expressed as fold changes in relative gene levels comparing to the housekeeping beta-actin gene. All values were expressed as mean ± SEM of three independent experiments. An apparent increase in TLR4 gene expression was observed in both gland cells and stromal cells in the secretory phase compared with that of similar cells derived from the proliferative phase of the menstrual cycle.