

Review Article

Toll-like receptor system and endometriosis

Khaleque Newaz Khan^{1*}, Michio Kitajima¹, Akira Fujishita², Masahiro Nakashima³,
Hideaki Masuzaki¹

¹Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences; ²Saiseikai Nagasaki Hospital, Nagasaki; ³Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki, Japan.

Running head: TLRs in endometriosis

* Address for Correspondence:

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

Abstract

Endometriosis is an estrogen-dependent chronic inflammatory condition associated with variable degrees of pelvic pain and infertility. Studies showed that the growth and progression of endometriosis continue even in ovariectomized animal. This indicates that besides ovarian steroid hormones, the growth of endometriosis can be regulated by innate immune system in pelvic environment. As a component of innate immune system, increased infiltration of macrophages has been described in the intact tissue and peritoneal fluid of women with endometriosis. Different immune cells and dendritic cells express Toll-like receptors (TLR) and exhibit functional activity in response to microbial products. In this review article, we discussed the role of Toll-like receptor (TLR) system in endometrium and endometriosis and outlined the involvement of cytokines/ endotoxin in causing adverse reproductive outcome. In the first part of this review article, the fundamentals of innate immune system, functional characteristics of TLRs, and signaling pathways of TLR4 are discussed for easy understanding by the readers.

Keywords: Innate immunity, TLRs, bacterial endotoxin, endometrium, endometriosis, infertility

1. Introduction

It is well recognized that innate and adaptive immune system are the two key branches that determine host protection throughout the female reproductive tract and at other mucosal surfaces, including the respiratory, gastrointestinal and urinary tracts. Our understanding of the innate immune system is a result, in large part, of the pioneering studies of Charles Janeway, who demonstrated that innate immunity covers many areas of host defense against pathogenic microbes.¹ During the last decade, investigations of the innate immune system have shown that microbial pathogens are recognized by Toll-like receptors (TLRs) that, in turn, regulate the activation of both innate and adaptive immunity.² Mammalian innate immune cells such as macrophages and dendritic cells can be activated by microbial components (non-self) such as endotoxin or lipopolysaccharide (LPS) from Gram-negative bacteria.

Analysis of the female reproductive tract indicates that the key cells of the innate and adaptive immune systems are present and functionally responsive to antigens.³ The innate immune system has evolved to recognize foreign structures that are not normally found in the host. It relies on conserved germ-line-encoded receptors that

recognize conserved pathogen-associated molecular patterns (PAMP) found in groups of microorganism.⁴ The pattern recognition receptors (PRR) of the host that recognize PAMP in female reproductive tract are expressed on the cells of the innate immune system. Toll-like receptors are one group of PRRs that are expressed on macrophages (M ϕ), dendritic cells, and as more recently shown, on neutrophils, natural killer cells, and on epithelial cells.³⁻⁵

Originally described over three hundred years ago, endometriosis is classically defined by the presence of endometrial glands and stroma in extrauterine locations.⁶ Basically endometriosis is an estrogen dependent disease mostly affecting women of reproductive age. Recently it has been demonstrated that besides hormonal regulation, both secondary and initial inflammatory mediators are known to involve in the growth of endometriosis.⁷⁻¹⁰ A number of literatures including ours have demonstrated the expression of TLRs in macrophages and other dendritic cells.⁸⁻¹³ In this review article, beginning with a fundamental concept of TLR system, we also discussed the source of initial inflammatory mediator, bacterial endotoxin or LPS, in intrauterine environment, its functional activity with TLR4 in eutopic and ectopic endometrium, and finally its

possible association with reproductive outcome in women with endometriosis.

2. Identification of the TLR Family:

After the characterization of the first mammalian TLR (TLR4), several proteins that are structurally related to TLR4 were identified and named Toll-like receptors.¹⁴ Mammalian TLRs comprise a large family consisting of at least 11 members. TLRs1-9 were found to be conserved between human and mouse. TLR10 is presumably functional in the human but non-functional in mouse. Similarly, mouse TLR11 is functional, but there is a stop codon in the human *TLR11* gene, which results in a lack of production of human TLR11.¹⁵

The cytoplasmic portion of TLRs shows high similarity to that of the interleukin (IL)-1 receptor family, and is termed a Toll/IL-1 receptor (TIR) domain. Despite this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-1 receptors possess an immunoglobulin-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain. Functionally, a critical role of TLR4 in the recognition of microbial component was initially characterized.¹⁶ Subsequently, it has been established that individual TLRs play important roles in

recognizing specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses.

Toll-like receptor 2 (TLR2) is essential in the recognition of microbial lipopeptides and peptidoglycan derived from Gram-positive bacteria. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR2 forms heterophilic dimers with TLR1 and TLR6, both of which are structurally related to TLR2.¹⁷ TLR4 is the receptor for LPS derived from the outer membrane of Gram-negative bacteria. TLR5 recognizes flagellin. TLR3 is implicated in the recognition of viral dsRNA associated with viral replication, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. Thus, polyriboinosinic:polyribocytidylic acid [poly (I:C)], which is a synthetic mimetic for dsRNA, can induce TLR3 signaling.¹⁸ TLR9 is essential in unmethylated (CpG) DNA recognition.⁴

3. Ligands of Toll-like Receptor 4 (TLR4):

3.1. Exogenous ligands. There are two types of ligands, exogenous and endogenous, for TLR4.¹⁹ As described above, TLR4 is an essential receptor for bacterial

endotoxin or LPS recognition. In addition to LPS, other exogenous ligands are F protein from respiratory syncytial virus, chlamydial heat shock protein 60 and taxol, a plant derived anticancer reagent that mimics the action of LPS in mice but not in humans.¹⁹

3.2. Endogenous ligands. Endogenous ligands of TLR4 comprise fibronogen, fibronectin, heparan sulphate, hyaluronic acid and heat shock proteins (Hsp) 60 and 70. However, all of these endogenous ligands require very high concentration to activate TLR4. It has been shown that contamination of LPS in Hsp70 preparation confers ability to activate TLR4. LPS is a very potent immuno-activator and accordingly, TLR4 can be activated by a very small amount of LPS, contaminating these endogenous ligand preparations.^{4,19-22} Therefore, we need careful attention in biological research using these endogenous ligands. The different TLRs and their corresponding ligands are described in

Table 1.

4. Signaling Pathways Triggered by TLR4:

Several lines of evidence indicate that all TLR signaling pathways are similar and elicit similar biological responses except TLR3.^{23,24} Lipopolysaccharide (LPS) is a potent activator of M ϕ and other dendritic cells. After being released into the blood

stream or other body fluids, LPS is immediately captured by LPS-binding protein (LBP) that delivers LPS to TLR4 or CD14. CD14 lacks a trans-membrane domain and so is incapable of transducing signals.²⁵ Both the positional cloning of the locus responsible for LPS hypo-responsiveness in C3H/HeJ mice and the generation of TLR4 knockout mice have shown that TLR4 is essential for LPS signaling.^{16,21} In addition, the interaction of LPS with TLR4 requires another molecule, MD-2, which associates with the extracellular domain of TLR4.

4.1. MyD88-dependent pathway. Once TLRs are activated, the intracellular signaling pathways are very similar between insects and mammals. In mammals, TLR4 signaling involves activation of one or more of the adaptor proteins. The adaptors relevant to TLR4 signaling are known as MyD88 (myeloid differentiation factor 88), TIRAP (TIR domain-containing adaptor protein), TRIF (TIR-domain containing-adaptor inducing IFN- β), and TRAM (TRIF-related adaptor molecule).^{4,26} Most TLRs act through MyD88 alone or through both MyD88 and TIRAP, which leads to the production of different pro-inflammatory cytokines. MyD88 is an adaptor molecule that recruits the kinase IRAK (IL-1 receptor-associated kinase) to the TLR4 receptor complexes after

stimulation with LPS. The lipopeptide activation of nuclear factor (NF)- κ B and MAP (mitogen-activated protein) kinases, as mediated by TLR2, is completely abolished in TLR2-depleted or MyD88-deficient M ϕ . By contrast, LPS activation of MAP kinases and NF- κ B remains intact in MyD88-deficient M ϕ . This indicates that LPS response is mediated by both MyD88-dependent and MyD88-independent pathways, each of which leads to the activation of MAP kinases and NF- κ B.

4.2. MyD88-independent pathway. The MyD88-dependent pathway is essential, however, for the inflammatory response mediated by LPS. The TIRAP has a crucial role in the MyD88-dependent signaling pathway shared by TLR2 and TLR4. Recent studies have shown that MyD88-independent pathway for TLR4 operates through different adaptor molecules, TRIF and TRAM, activates interferon (IFN) regulatory factor 3 (IRF-3), up-regulates co-stimulatory molecules and leads to the subsequent induction of type I interferon such as IFN- β , nitric oxide synthase (iNOS) and IFN-inducible protein (IP-10).^{4,26} It is important to remember that in addition to activation of IRF3, MyD88-independent pathway also elicits delayed activation of NF- κ B. Studies are still limited with MyD88-independent pathway. TLR4 signaling

pathways are shown in **Figure 1**.

Unlike other TLRs, TLR3 uses only one adaptor protein, TRIF, whose activation leads to IRF3 translocation to the nucleus. IRF3 dimerizes and enters the nucleus where it binds to interferon-sensitive response element (ISRE) motifs and induces the expression of type I interferons, IFN- α and IFN- β .^{23,24} Female reproductive tract and placenta may become exposed to viruses in addition to bacterial or fungal infection, which may pose a substantial threat to reproductive outcome or embryo/fetus well-being. Although studies are limited, it is important to determine the type of virus and whether the engagement of TLR3 with viral dsRNA could induce production of factors necessary to generate an antiviral response. In fact, TLR3 expression has been demonstrated in the epithelial cells of vagina, uterine cervix, endometrium, fallopian tubes and also in placenta.^{11,27}

5. TLR system in endometriosis

5.1. TLRs in eutopic endometrium. For most of the reproductive cycle in humans and animals, the uterus is thought to be sterile or at least clear of pathogenic bacteria, but it is readily contaminated with bacteria during sexual intercourse and around

the time of parturition. In fact, the upper genital tract is vulnerable to the spread of microorganisms from the lower genital tract, resulting in the development of infectious diseases such as endometritis and salpingitis. In fact, an enormous number of Gram-negative and Gram-positive microbes are present in vaginal cavity (**Table 2**). All these microbes reside in vaginal cavity as normal vaginal flora and may cause genitourinary infections upon ascending migration.²⁷

In recent years increasing attention has been paid to innate immunity, the primary defense system against pathogens. *Escherichia coli* (*E.coli*) are the most commonly isolated pathogenic bacteria from clinical uterine diseases in cattle²⁸ and also in human vaginal cavity.²⁹ The ascending migration of *E.coli* towards endometrial cavity is possible that may cause contamination of endometrium. The endometrium provides a barrier against infection and an opportunity to detect these bacteria by innate immune receptors. Toll-like receptors were first identified on immune cells but have since been identified on other cell types including endometrium.³⁰ In the human endometrium, nine TLRs are identified at the protein and mRNA level including TLR4.^{12,31-33} Engagement of these receptors initiates a signaling cascade stimulating the production of immune

mediators that orchestrate the immune response to clear the infection. It is the principal role of TLR4 to detect LPS, although signaling through TLR4 also requires accessory molecules such as LBP, CD14 and MD2.

As a component of innate immune system, an increase in the infiltration of M ϕ was found in normal endometrium and also in the endometrium of women with different reproductive diseases such as endometriosis, adenomyosis and uterine leiomyoma.³⁴⁻³⁶ The expression of TLR4 mRNA and protein was detected in M ϕ , endometrial epithelial cells and stromal cells.^{10,31,32} RT-PCR analysis also demonstrated the expression of CD14, MD2 and MyD88 mRNA in both endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs).³² The expression levels of TLR4, CD14, and MD2 appeared to be higher in ESCs compared with that in EECs. However, the expression levels of MyD88 were similar between ESCs and EECs.

Treatment of endometrial stromal cells with LPS significantly increased the production of a number of macromolecules, such as hepatocyte growth factor (HGF), vascular endothelial cell growth factor (VEGF), interleukin (IL)-6, IL-8 and tumor necrosis factor alpha (TNF α) in a dose-dependent fashion.^{32,37,38} A significantly more

growth promoting effect of LPS was observed on endometrial cells derived from women with endometriosis when compared with similar cells derived from control women.^{37,38}

The stimulatory effect of LPS was inhibited by the addition of neutralizing antibodies for TLR4 and also by an LPS antagonist, polymyxin B.¹⁰ This indicates that M ϕ , ESCs and EECs express TLR4 and respond to LPS through TLR4. In fact, we recently demonstrated that both ESCs and EECs were able to significantly proliferate in response to LPS and this growth promoting effect of LPS was abrogated after pretreatment of cells with anti-TLR4 antibody.^{8,10,39} Since, there are other exogenous and endogenous ligands for TLR4 in addition to LPS, we presume that blocking of TLR4 alone is more effective in order to suppress inflammatory response in pelvic environment and cell growth.

A recent study³² demonstrated that LPS was able to stimulate TLR4- and CD14-mediated increased production IL-8 by ESCs. This effect of LPS was associated with the activation of NF- κ B as examined by nuclear translocation of NF- κ B in ESCs. On the other hand, LPS alone did not stimulate IL-8 secretion in EECs. However, LPS did stimulate IL-8 secretion from EECs in the presence of soluble CD14. These findings indicate that TLR4 system might represent local immunity in the human endometrium

with different modes of TLR4 actions between ESCs and EECs.

5.2. TLRs in ectopic endometrium. We presume that an innate immune system and ovarian steroid hormones may participate either alone or in an orchestrated fashion in the growth regulation of endometriosis. The different macromolecules as secreted by M ϕ in the pelvic environment are believed to enhance the growth of endometriosis. However, the initial inflammatory mediator that stimulates M ϕ for the production of different cytokines and growth factors was poorly described. We reported that bacterial endotoxin (LPS), could be a potential inflammatory mediator of M ϕ stimulation and consequent production of different cytokines and growth factors, such as HGF, VEGF, IL-6 and TNF α in pelvic environment.³⁷ This LPS and together with LPS-induced secondary inflammatory mediators are possibly involved in the growth of endometriosis in an autocrine or paracrine mechanism.³⁷

There was no information until now about the presence of bacterial endotoxin in pelvic environment. We examined endotoxin concentration for the first time in the menstrual fluid (MF) and peritoneal fluid (PF) of women with or without endometriosis. We found that endotoxin (LPS) concentration in MF/PF was significantly higher in

women with endometriosis than that in non-endometriosis. The expression pattern of TLR4 in M ϕ , endometrial cells and endometriotic cells was identical between women with endometriosis and non-endometriosis in the proliferative phase but this expression pattern appeared to be higher in the secretory phase of the menstrual cycle.^{10,12,33} The production of HGF, VEGF, IL-6 and TNF α by LPS-treated peritoneal M ϕ was significantly higher in women with endometriosis than that in women without endometriosis. This was evident at both protein and mRNA level. The blocking of TLR4 after pretreatment of M ϕ with anti-TLR4 antibody significantly reduced the production of all these cytokines.^{8,10,39} The addition of culture media from TLR4-blocked macrophages caused significant suppression in the growth of endometrial and endometriotic cells comparing to that of TLR4 non-blocking macrophages. The direct application of LPS also promoted the growth of endometriotic cells derived from women with peritoneal endometriosis and was suppressed after pretreatment of cells with anti-TLR4 antibody.¹⁰

Similar line of study⁴⁰ with ESCs derived from chocolate cyst linings of the ovary demonstrated that LPS-stimulated ESCs produced significant amount of TNF α

and IL-8 and addition of LPS to ESCs promoted significant cell proliferation. This stimulating effect of LPS was abrogated after treatment with NF- κ B inhibitor.⁴⁰ This indicates that as an initial inflammatory mediator, functional activity of LPS is regulated by both TLR4 at the receptor level on cell surface and by NF- κ B at the nucleus. These results also suggested that a substantial amount of endotoxin in MF/PF is involved in pelvic inflammation and may promote TLR4/ NF- κ B-mediated growth of endometriosis. Therefore, targeting TLR4 or NF- κ B could be a new therapeutic strategy to reduce inflammatory reaction in pelvic environment and prevent consequent growth of endometriosis.

There might be two mechanisms for the residual accumulation of bacterial endotoxin in pelvic environment: (a) translocation of *E.coli* or endotoxin from the gut through enterocytes and their entry into the pelvic cavity as demonstrated by Alexander et al.,⁴¹ (b) contamination of menstrual blood by *E.coli* after ascending migration from vagina. Since endometriosis is a product of retrograde menstruation and LPS is a cell wall extract of *E.coli*, we demonstrated that menstrual blood of women with endometriosis was highly contaminated with *E.coli* than that in control women.¹⁰ We detected colony

formation of *E.coli* in menstrual blood and this was significantly higher in women with endometriosis than that in non-endometriosis.¹⁰ The contamination of menstrual blood with *E.coli* was associated with a parallel increase in the level of endotoxin in the menstrual blood. Our findings suggested that contamination of menstrual blood with *E.coli* in women with endometriosis could be a constant source of bacterial endotoxin in peritoneal fluid due to periodic retrograde menstrual flow and this cyclic event may initiate TLR4-mediated growth of endometriosis.

As a mechanistic basis of *E.coli* contamination in menstrual blood, We recently demonstrated that higher prostaglandin E₂ (PGE₂) levels in the MF/PF of women with endometriosis was involved in the bacterial growth such as *E.coli* in a bacteria culture system.⁴² This effect of PGE₂ on bacteria may be contributed by its direct growth promoting effect on *E.coli* or by its indirect immunosuppression effect on peripheral blood lymphocytes.⁴² The decreased expression of antimicrobial peptides in intrauterine or intra-vaginal luminal epithelium may be involved in bacterial contamination of menstrual blood in women with endometriosis.⁴³ We postulate that a possible sub-clinical vaginal infection or changes in intra-uterine defense against microbes in women with

endometriosis might be involved in the bacterial contamination of menstrual blood and consequent participation of an LPS/TLR4 cascade in the growth of endometriosis.^{10,42,43}

6. Inflammation, stress reaction and TLR4 in endometriosis

In addition to pelvic inflammation, endometriosis may equally produce a stress reaction and release endogenous heat-shock proteins in pelvic environment as a result of tissue damage, tissue invasion and by inflammatory reaction itself. A wide variety of stressful stimuli, such as heat shock, ultraviolet radiation, viral or bacterial infections, internal physical stress, chemical stress and pelvic inflammation, induce an increase in the intracellular synthesis of stress-induced proteins, such as heat shock proteins (Hsp).⁴⁴⁻⁴⁶ The so-called ‘danger theory’ states that antigen presenting cells can be activated by endogenous substances released by damaged or stressful tissues⁴⁷ and this effect of Hsps has been reported to be mediated by TLR4 either alone or in combination with LPS.³⁹

We recently demonstrated the release of a variable amount of endogenous Hsp70 by the different peritoneal lesions and eutopic endometria of women with endometriosis and that this locally produced Hsp70 might be responsible for

TLR4-mediated induction of inflammatory reaction and direct promotion in the growth of endometriosis.³⁹ Although, polymyxin B, a potent LPS antagonist was able to suppress LPS-mediated growth of endometrial cells derived from women with endometriosis as reported previously.^{32,39} polymyxin B was unable to suppress combined LPS- and Hsp70-mediated growth of endometriosis. In contrast, the growth promoting effect of combined LPS and Hsp70 was significantly suppressed when the biological function of TLR4 was blocked with anti-TLR4 antibody.⁴⁸ This indicates that LPS- and Hsp70-mediated inflammatory reaction and growth of endometriosis may be mediated by TLR4 in pelvic environment.

Other potential contribution factors for tissue stress reaction include oxidative stress resulting from excessive iron accumulation in endometriotic fluid, because endometriotic lesions including chocolate cyst and blood-filled opaque red lesions are hemorrhagic during menstruation.^{49,50} In addition to their involvement in atherosclerosis, neurodegeneration, cancer and aging,⁵¹ excessive reactive oxygen species (ROS) production or oxidative stress might be associated with endometriosis. Recently it has been demonstrated that in addition to the effects of endogenous danger signals via TLRs,

tissue oxidative stress itself may promote NF-kB-mediated or TLR4-mediated growth of endometriosis.⁵² In fact, LPS itself has the capacity to produce ROS by macrophages. These findings are consistent with the understanding that LPS, endogenous danger signals and oxidative stress may promote the onset and progression of endometriosis after activation of TLRs and/or NF-kB signaling.

7. Inflammation, ovarian steroid and TLR4 in endometriosis

Basically endometriosis is an estrogen-dependent disease and induces an inflammatory reaction in pelvic environment. An abundant number of literatures have already demonstrated individual effect of estrogen and effect of initial or secondary inflammatory mediators in the growth regulation of endometriosis.⁵³⁻⁵⁶ Therefore, it is important to know the combined effect of estrogen and inflammation in the growth of endometriosis.

Recently, we reported that macrophage-mediated production of HGF/VEGF/IL-6/TNF α in response to ovarian steroids was further enhanced after treatment with LPS.⁵⁵ A synergistic effect was observed between E₂ and LPS on the proliferation of eutopic and ectopic endometrial stromal cells when compared with their

single treatment. This effect of E₂+LPS on cell growth was markedly abrogated after pretreatment of cells with anti-TLR4 antibody and ICI, an ER antagonist.^{8,55,57} Our findings suggest that E₂ exhibits pro-inflammatory response and an immuno-endocrine cross-talk between estrogen and endotoxin in pelvic environment may be involved in additive inflammatory response in pelvic environment and growth of endometriosis. Another published report on this issue is in consistent with our findings.⁵⁸

8. Effect of cytokines/bacterial endotoxin on reproductive outcome

The ultimate fates of women who suffer from endometriosis are impairment in quality of life and reduction in the rate of fertilization, implantation and finally failure to achieve pregnancy.^{59,60} The purpose of current medical and surgical therapy for the women with endometriosis is to remove endometriotic lesions, to relieve periodic or non-periodic pain experience and to establish pregnancy. Several proposed mechanisms of implantation failure in women with endometriosis have been reported elsewhere including progesterone resistance, alteration in PR-A to PR-B ratio.⁶¹ Endometriosis-associated infertility can be explained by one of the several mechanisms as shown in **Figure 2**.

The increased infiltration of macrophages and other immune cells may have two-fold effects on endometrial bed in women with endometriosis. One, direct phagocytosis of implanting embryos; secondly, indirect impairment in the process of implanted blastocyst. These hazardous effects of M ϕ can be contributed by producing some biological mediators such as ROS or by inducing humoral immune response.^{60,62,63}

A moderate to severe inflammatory reaction in pelvic environment leads to the formation of tubo-ovarian adhesion or peri-tubal adhesion finally resulting in narrowing or occlusion of the Fallopian tube.⁶⁴ On the other hand, bacterial endotoxin (LPS) derived from Gram-negative bacteria may directly cause endometrial or tubal damage. Endotoxin has been found to be deleterious on pre-implantation stage embryos.⁶⁵ The presence of endotoxin in IVF culture media results in high rate of polyspermy, decreased embryo cleavage rate and blastocyst formation in human and bovine species. Endotoxins also possess the capacity to induce apoptosis of cells impairing sperm motility and induce spermicidal activity.⁶²⁻⁶⁵ A recent ART clinical trial has demonstrated that pregnancy rate after IVF-ET was significantly higher in women with an endotoxin level of <200 pg/ml in menstrual fluid, than that in women with an endotoxin level of >200 pg/ml.⁶⁶

In addition to female, bacterial infections of the genital tract are one of the most serious causes of infertility in male. A recent study detected Gram-negative bacteria factor, LPS and Gram-positive bacteria factor, peptidoglycan in human semen and demonstrated expression of TLR4 and TLR2, peptidoglycan receptor, in human and mouse sperm.⁶⁷ They found that addition of endotoxin in the absence of leukocytes directly and significantly reduced the motility and increase the apoptotic rate of both human and mouse sperm and suppressed fertilization by sperm both in vivo and in vitro.⁶⁷ These findings further strengthened the detrimental effect of bacterial endotoxin on reproductive outcome.

Many of the biological effects of bacterial endotoxin are mediated by pro-inflammatory cytokines such as IL-1, IL-6, and TNF α . One recent study demonstrated that adding recombinant IL-6 to culture media suppressed the rate of blastocyst formation in mouse embryos and reduced the percentage of motile human spermatozoa.⁶⁸ Higher concentrations of TNF α possess apoptosis- and necrosis-inducing activity on a variable type of cells including sperm, ova and endometrial cells.^{65,69}

Macrophages also produce increased concentrations of ROS (OH^- , O_2^- and H_2O_2) in response to bacterial endotoxin. The increased generation of ROS at the tissue level induces a wide range of biological activity such as lipid peroxidation, protein denaturation, inactivation of enzymes and decomposition of cellular DNA.⁷⁰ In this way, ROS may cause cellular and tissue damage. These unwanted effects of ROS may cause impairment of ova or sperm function. Bacterial endotoxin-induced increase in ROS production may also cause caspase-mediated apoptosis.⁶⁹ This apoptosis-inducing effect of ROS may result in endometrial or tubal epithelial damage, impairment in fertilization and sperm motility.^{62,63}

9. Summary and perspective

We now know that innate immunity plays an important role in the initiation of immune response in pelvic environment. A number of widely accepted mechanisms involved in the development or pathogenesis of endometriosis are summarized and is shown in **Figure 3**. The production of pro-inflammatory cytokines and growth of endometriosis in pelvic environment can be regulated by innate immune system. We proposed for the first time a new concept “bacterial contamination hypothesis” in endometriosis and involvement of LPS/TLR4 cascade in the growth regulation of

endometriosis. Our results suggest that a substantial amount of endotoxin in peritoneal fluid due to reflux of menstrual blood is involved in pelvic inflammation and may promote TLR4-mediated growth of endometriosis. Targeting bacterial endotoxin or TLR4 or NF- κ B could be useful as a therapeutic strategy to suppress pelvic inflammation and growth of endometriosis with consequent improvement in the quality of life and fertility rate of women who suffer from this enigmatic disease. Our ongoing study targeting to find the evidence of a sub-clinical infection within vaginal cavity of women with endometriosis may hold new therapeutic potential in addition to conventional estrogen suppressing agent. A complete understanding of the mechanisms of innate immunity and TLR system will be helpful for the future development of innovative therapies for the manipulation of endometriosis and other reproductive diseases.

10. Acknowledgements

We thank 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.

11. Disclosure

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). The authors report no conflict of interest related to this review article.

12. References

1. Janeway CAJ: Approaching the Asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 1989;54:1-13.
2. Janeway CAJ and Medzhitov R: Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.
3. Reichhart JM: TLR5 takes aim at bacterial propeller. *Nat Immunol.* 2003;4:1159-1160.
4. Takeda K and Akira S: Toll-like receptors in innate immunity. *Int. Immunol.* 2005;17:1-14.
5. Wira CR, Fahey JV, Sentman CL, Pioli PA, and Shen L: Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol Reviews* 2005;206:306-335.

6. Burney RO and Giudice LC. Pathogenesis and pathophysiology of endometriosis.

Fertil Steril 2012;98(3):511-519.

7. Attar E and Bulun SE: Aromatase and other steroidogenic genes in endometriosis:

translational aspects. *Hum Reprod Update* 2006;12(1):49-56.

8. Khan KN, Kitajima M, Hiraki H, Fujishita A, Sekine I, Ishimaru T, Masuzaki H.

Immunopathogenesis of pelvic endometriosis: role of hepatocyte growth factor, macrophages and ovarian steroids. *Am J Reprod Immunol* (review), 2008;60:383-404.

9. Khan KN, Kitajima M, Hiraki H, Fujishita A, Sekine I, Ishimaru T, Masuzaki H.

Toll-like receptors in innate immunity: role of bacterial endotoxin and toll-like receptor 4 (TLR4) in endometrium and endometriosis. *Gynecol Obstet Invest* (review) 2009;68:40-52.

10. Khan KN, Kitajima M, Hiraki K, Yamaguchi N, Katamine S, Matsuyama T, Fujishita

A, Nakashima M, Ishimaru T, Masuzaki H. Escherichia coli contamination of menstrual blood and effect of bacterial endotoxin on endometriosis. *Fertil Steril* 2010; 94(7):2860-2863.

11. Fazeli A, Bruce C, Anumba DO: Characterization of Toll-like receptors in the

female reproductive tract in humans. *Hum Reprod* 2005,20(5),1372-1378.

12. Aflatoonian R, Tuckerman E, Elliot SL, Bruce C, Aflatoonian A, Li TC, Fazeli A:

Menstrual cycle-dependent changes of toll-like receptors in endometrium. *Hum Reprod* 2007,22,586-93.

13. Hirata T, Osuga Y, Hamasaki K, Hirota Y, Nose E, Morimoro C et al., Expression of

Toll-like receptor 2, 3, 4, and 9 in the human endometrium during the menstrual cycle. *J Reprod Immunol* 2007,74:53-60.

14. Rock FL, Hardiman G, Timans JC, Kastelein RA and Bazan JF: A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl Acad. Sci.* 1998;95:588.

15. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA and Ghosh S: A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004;303:1522-1526.

16. Poltorak A et al: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutation in *Tlr4* gene. *Science* 1998;282:2085-2088.

17. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K and Akira S: Differential roles of TLR2 and TLR4 in recognition of gram-negative and

- gram-positive bacterial cell wall components. *Immunity* 1999;11:443-451.
18. Nasu K, Itoh H, Yugae A, Nishida M, Narahara H: Human oviductal epithelial cells express Toll-like receptor 3 and respond to double-stranded RNA: Fallopian tube-specific mucosal immunity against viral infection. *Hum Reprod* 2007;22:356-361.
19. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, and Schwartz DA: Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med* 2002;347:185-192.
20. Akira S and Takeda K: Toll-like receptor signaling. *Nat Rev Immunol.* 2004;4:499-511.
21. Hoshino K, Takeuchi O, Kawai T, Sanjo T, Ogawa T, Takeda Y, Takeda K and Akira S: Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the LPS gene product. *J Immunol.* 1999;162:3749-3752.
22. Wallin RPA, Lundqvist A, More SH, von Bonin A, Kiessling R, and Ljunggren H-G: Heat-shock proteins as activators of the innate immune system. *TRENDS in Immunol*

2002;**23(3)**:130-135.

23. McCoy CE and O'Neill LAJ: The role of toll-like receptors in macrophages.

Frontiers in Bioscience 2008;13:62-70.

24. Parker LC, Prince LR, Sabroe I: Translational mini-review series on toll-like receptors: networks regulated by toll-like receptors mediate innate and adaptive immunity. *Clin Exp Immunol* 2007;147:199-207.

25. Akira S: Mammalian Toll-like receptors. *Curr. Opin. Immunol.* 2003;15:5-11.

26. Check W: Innate immunity depends on Toll-like receptors. *ASM News*

2004;70:317-322.

27. Abrahams VM, Schaefer TM, Fahey JV, Visintin I, Wright JA, Aldo PB, Romero R,

Wira CR, Mor G: Expression and Secretion of antiviral factors by trophoblast cells

following stimulation by the TLR-3 agonist, Poly (I:C). *Hum Reprod*

2006;21:2432-2439.

28. Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU and Dobson H: Influence of

uterine bacterial contamination after parturition on ovarian dominant follicle

selection and follicle growth and function in cattle. *Reproduction* 2002;123:837-845.

29. Larsen S and Galask RP: Vaginal microbial flora: practical and theoretic relevance. *Obstet Gynecol* 1980;55:100S-113S.
30. Eriksson M, Measows SK, Basu S, Mselle TF, Wira CR, Sentman CL: TLRs mediate IFN- γ production by human uterine NK cells in endometrium. *J Immunol* 2006;176:6219-6224.
31. Young SL, Lyddon TD, Jorgenson RL, and Misfeldt ML: Expression of Toll-like receptors in human endometrial epithelial cells and cell lines. *Am J Reprod Immunol* 2004;52:67-73.
32. Hirata T, Osuga Y, Hirota Y, Koga K, Yoshino O, Harada M, Morimoto C, Yano T, Nishii O, Tsutsumi O, Taketani Y: Evidence for the presence of Toll-like receptor 4 system in the human endometrium. *J Clin Endocrinol Metab* 2005;90:548-556.
33. Fazeli A, Bruce C, Anumba DO: Characterization of Toll-like receptors in the female reproductive tract in humans. *Hum Reprod* 2005,20(5),1372-78.
34. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T: Differential macrophage infiltration in early and advanced endometriosis and adjacent peritoneum. *Fertil. Steril.* 2004;81:652-661.

35. Khan KN, Kitajima M, Hiraki K, Fujishita A, Sekine I, Ishimaru T, Masuzaki H. Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy. *Hum Reprod*. 25(3):642-53, 2010.
36. Miura S, Khan KN, Kitajima M, Hiraki K, Moriyama S, Masuzaki H, Samejima T, Fujishita A, Ishimaru T: Differential infiltration of macrophages and prostaglandin production by different uterine leiomyomas. *Hum Reprod* 2006;21(10):2545-2554.
37. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Kohno T, Sekine I, Matsuyama T, Ishimaru T: Regulation of hepatocyte growth factor by basal and stimulated-macrophages in women with endometriosis. *Hum Reprod* 2005;20:49-60.
38. Khan KN, Kitajima M, Fujishita A, Sekine I, Ishimaru T, Masuzaki H: Multifunctional role of hepatocyte growth factor (HGF) in the development of pelvic endometriosis. *WES e-journal* (review) 2008 (July):13-20.
39. Khan KN, Kitajima M, Imamura T, Hiraki K, Fujishita A, Sekine I, Ishimaru T, Masuzaki H: Toll-like receptor 4 (TLR4)-mediated growth of endometriosis by human heat shock protein 70 (Hsp70). *Hum Reprod* 2008;23:2210-2219.

40. Iba Y, Harada T, Horie S, Deura I, Iwabe T, Terakawa N. Lipopolysaccharide-promoted proliferation of endometriotic stromal cells via induction of tumor necrosis factor α and interleukin-8 expression. *Fertil Steril* 2004;82(suppl 3):1036-1042.
41. Alexander JW, Boyce ST, Babcock GF, Gianotti L, Peck MD, Dunn DL, Pyles T, Childress CP and Ash SK: The Process of microbial translocation. *Ann. Surg.* 1990;212:496-511.
42. Khan KN, Kitajima M, Yamaguchi N, Fujishita A, Nakashima M, Ishimaru T, Masuzaki H. Role of prostaglandin E2 in bacterial growth in women with endometriosis. *Hum Reprod* 2012;27(12):3417-3424.
43. Valore EV, Park CH, Quayle AJ, Willee KR, McCray Jr. PB, Ganz T. Human β -defensin-1: an antimicrobial peptide of urogenital tissues. *J Clin Invest* 1998;101:1633-1642.
44. Asea A, Krafft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finger RW, Koo GC, Calderwood SK. HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. *Nat. Med.*

2000;6:435-442.

45. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002;277:15028-15034.

46. Zugel U and Kaufmann SH. Immune response against heat-shock proteins in infectious diseases. *Immunobiology* 1999;201:22-35.

47. Matzinger P. An innate sense of danger. *Semin Immunol* 1998;10:399-415.

48. Inoue T, Khan KN, Kitajima M, Hiraki H, Fujishita A, Masuzaki H. Cross-talk between inflammation and stress reaction in Toll-like receptor 4-mediated growth of endometriosis. *Proceedings of the 11th World Congress on Endometriosis* 2011;p204.

49. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T. Higher activity by opaque endometriotic lesions than non-opaque lesions in women with endometriosis. *Acta Obstet Gynecol Scand* 2004;83:375-382.

50. Burney ROLathi RB. Menstrual bleeding from an endometriotic bleeding. *Fertil Steril* 2009;91:1926-1927.

51. Kalmar B, Greensmith L. Induction of heat shock proteins for protection against

oxidative stress. *Adv Drug Deliv Rev* 2009;61:310-318.

52. Kajihara H, Yamada Y, Kanayama S, Furukawa N, Noguchi T, Haruta S, Yoshida S, Sado T, Oi H, Kobayashi H. New insights into the pathophysiology of endometriosis: from chronic inflammation to danger signals. *Gynecol Endocrinol* 2011;27(2):73-79.

53. Beagley KW and Gockel CM: Regulation of innate and adaptive immunity by the female sex hormones estradiol and progesterone. *FEMS Immunol Med Microbiol* 2003;38:13-22.

54. Herath S, Fischer FD, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE and Sheldon IM: Expression and Function of Toll-like receptor 4 in the endometrial cells of the uterus. *Endocrinology* 2006;147:562-570.

55. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Matsuyama T, Ishimaru T: Estrogen and progesterone receptor expression in macrophages and regulation of hepatocyte growth factor by ovarian steroids in women with endometriosis. *Hum Reprod* 2005;20:2004-2013.

56. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Hiraki K, Sekine I, Matsuyama T, Ishimaru T: Interleukin-6 and tumor necrosis factor α -mediated expression of

- hepatocyte growth factor by stromal cells and its involvement in the growth of endometriosis. *Hum Reprod* 2005;20:2715-2723.
57. Khan KN, Kitajima M, Hiraki K, Inoue T, Fujishita A, Ishimaru T, Masuzaki H. Combined effect of estrogen and endotoxin on pelvic inflammation and growth of endometriosis. *64th Proceedings of Acta Obstretica et Gynecologica Japonica* 2012;p813.
58. Cakmak H, Guzeloglu-Kayisli O, Kayisli UA, Arici A. Immune-endocrine interactions in endometriosis. *Frontiers in Bioscience* 2009;E1:429-443.
59. D'Hooghe TM, Debrock S, Hill JA, Meuleman C: Endometriosis and subfertility: is the relationship resolved? *Sem Reprod Med* 2003;21(2):243-254.
60. Tomassetti C, Meuleman C, Pexsters A, Mihalyi A, Kyama C, Simsa P, D'Hooghe TM: Endometriosis, recurrent miscarriage and implantation failure: is there an immunological link? *Reprod BioMed Online* 2006;13(1):58-64.
61. Cakmak H, Taylor HS. Implantation failure: molecular mechanisms and clinical treatment. *Hum Reprod Update* 2011;17:242-253.
62. Agarwal A, Gupta S, and Sharma R: Oxidative stress and its implications in female infertility-a clinician's perspective (review article). *Reprod BioMed Online*

2005;11(5):641-650.

63. Gupta S, Agarwal A, Krajcir N, and Alvarez JG: Role of oxidative stress in endometriosis. *Reprod BioMed Online* 2006;13(1):126-134.
64. Adamson GD and Pasta DJ: Surgical treatment of endometriosis-associated infertility: meta-analysis compared with survival analysis. *Am J Obstet Gynecol* 1994;171:1488-1504.
65. Deb K, Chatturvedi MM and Jaiswal YK: Gram-negative bacterial endotoxin-induced infertility: a bird eye view. *Gynecol Obstet Invest* 2004;57:224-232,
66. Kamiyama S, Teruya Y, Nohara M, and Kanzawa K: Impact of detection of bacterial endotoxin in menstrual effluent on the pregnancy rate in in vitro fertilization and embryo transfer. *Fertil Steril* 2004;82:788-792.
67. Fujita Y, Mihara T, Okazaki T, Shitanaka M, Kushino R, Ikeda C, Negishi H, Liu Z, Richards JS, Shimada M. Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. *Hum Reprod* 2011;26:2799-2806.
68. Harada T, Ohata Y, Deura I, Taniguchi F, Iwabe T, Terakawa N: Serum cytokine

levels are elevated in patients with endometriosis. *WES e-journal* 2008 (July);5-8.

69. Eley A, Pacey AA, Galdiero M, Galdiero M, and Galdiero F: Can *Chlamydia*

trachomatis directly damage your sperm? *Lancet* 2005;5:53-57.

70. Ota H, Igarashi S, Hatazawa J, Tanaka T: Endometriosis and free radicals. *Gynecol*

Obstet Invest 1999;48(suppl 1):29-35.

Figure Legends

Figure 1. TLR4 signaling pathway and is reproduced with the permission of Dr. Shizuo Akira of Osaka University. TLR4 signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLR, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF 6, leading to the activation of I κ B kinase (IKK) complex. The IKK complex phosphorylates I κ B, resulting in nuclear translocation of NF- κ B which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3 and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN- β are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Atypical IKKs mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

Figure 2. Shows the detrimental effects of different cytokines being produced by infiltrated macrophages and endometrial/endometriotic tissues and of bacterial endotoxin on reproductive outcome.

Figure 3. Shows three most widely accepted proposals on the development or growth regulation of endometriosis. Our proposed concepts on the role of bacterial endotoxin in Toll-like receptor (TLR) 4-mediated growth of endometriosis and the crosstalk between estrogen and lipopolysaccharide (LPS) in the development, growth/persistence and progression of endometriosis is shown here.

Figure 1

TLR4 Signaling Pathway

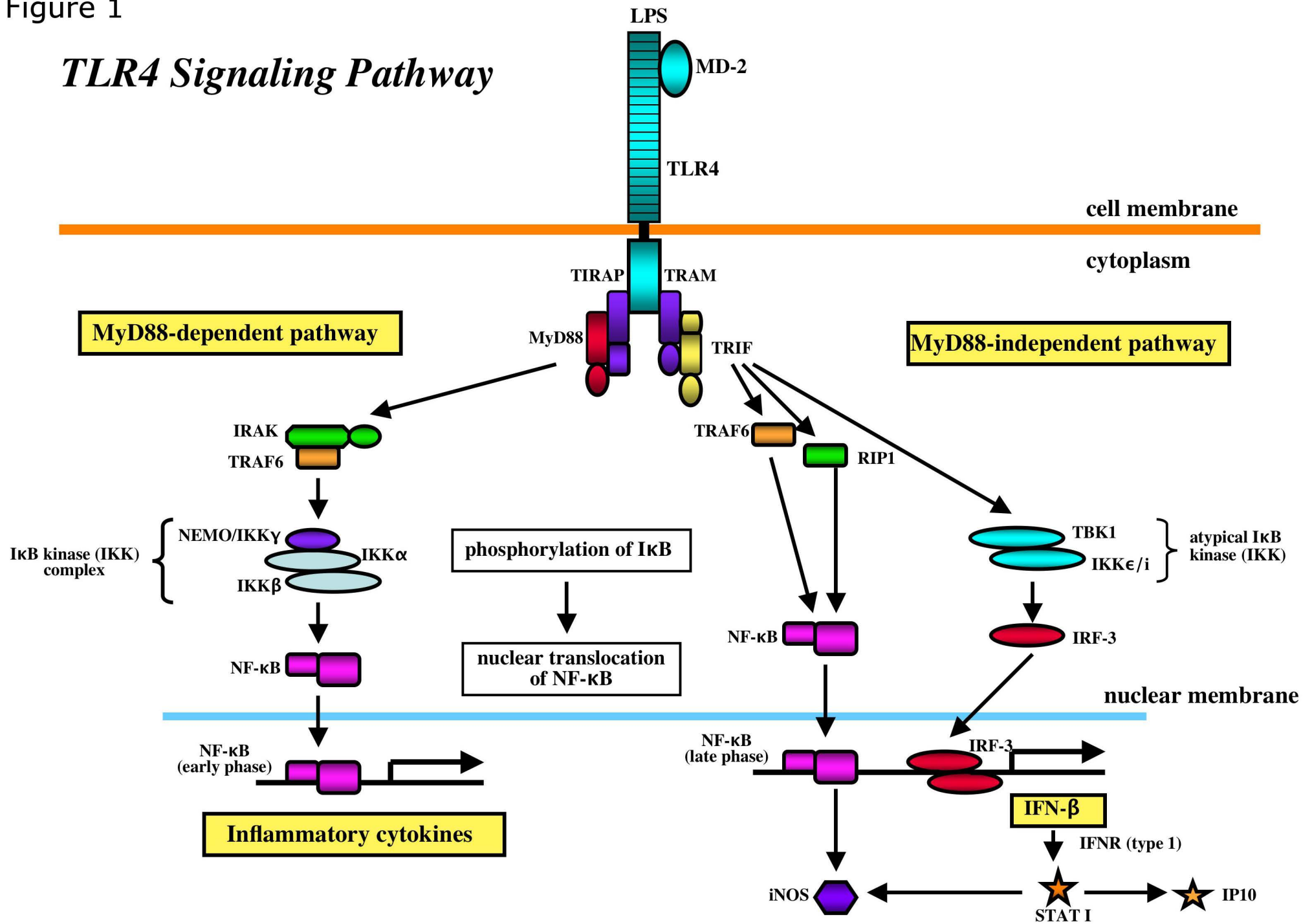


Figure 2

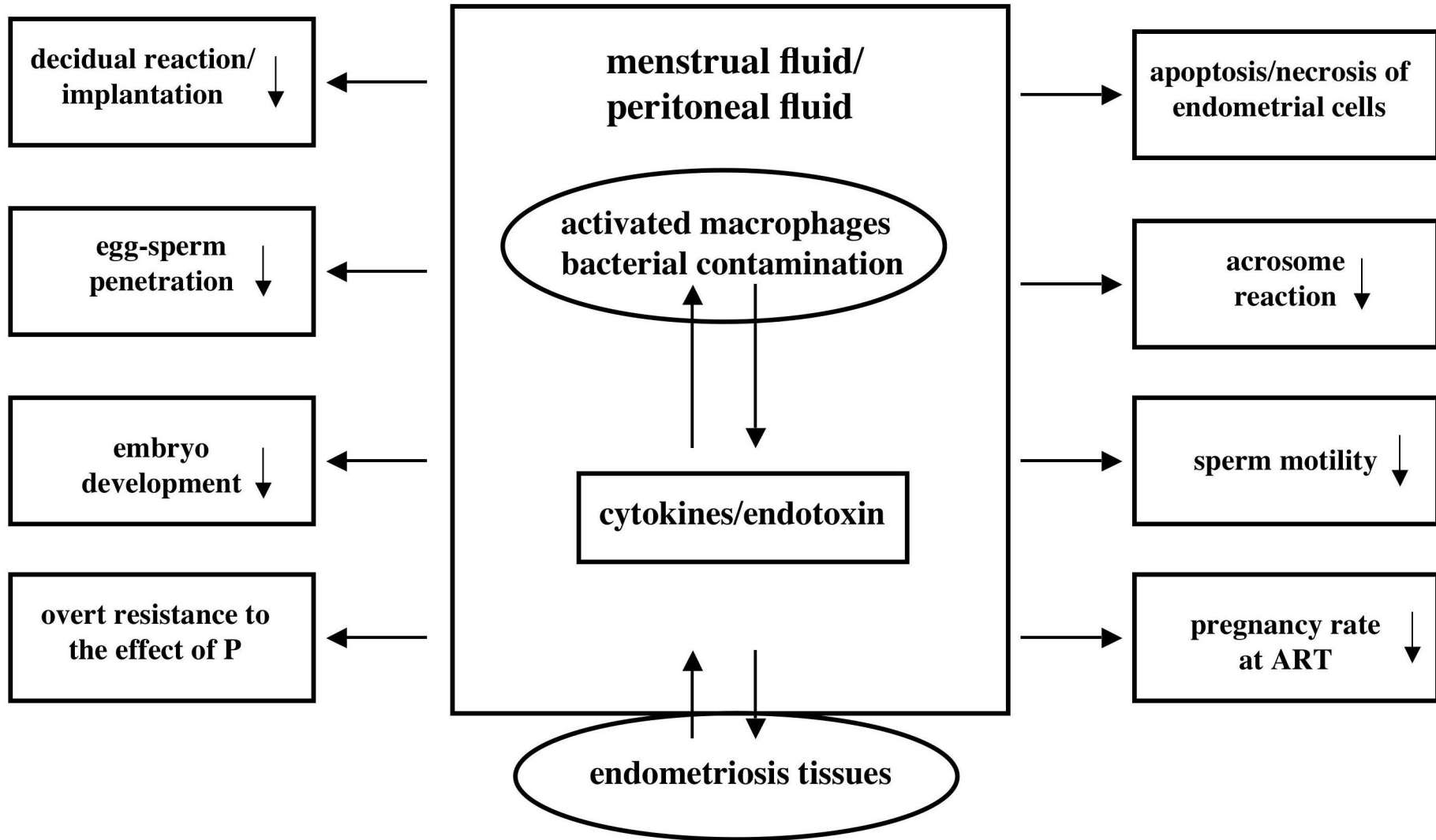


Figure 3

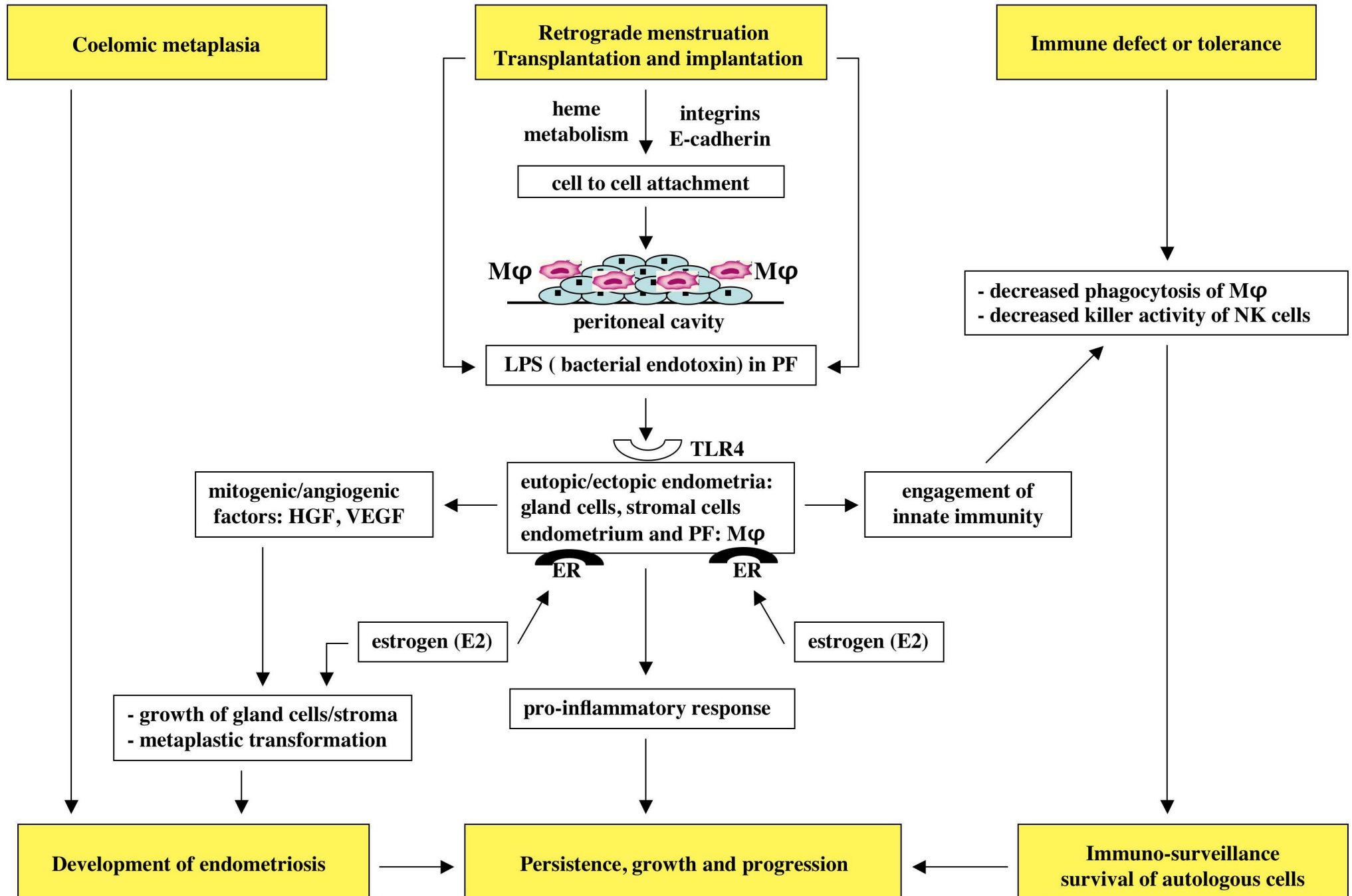


Table 1. Toll-like receptors (TLRs) and their ligands.

Receptors	Ligands	Origin of ligands
TLR1	Triacyl lipopeptide	Bacteria and mycobacteria
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Porins Atypical lipopolysaccharide Zymosan	Various pathogens Gram-positive bacteria Gram-positive bacteria Neisseria Porphyromonas gingivalis Fungi
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide Taxol Fusion protein Heat-shock protein 60 Heat-shock protein 60/70/90 Fibronectin Hyaluronic acid Heparan sulphate Fibrinogen	Gram-negative bacteria Plants (<i>Taxus brevifolia</i>) Respiratory syncytial virus <i>C. trachomatis/pneumoniae</i> Host Host Host Host Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides Zymosan	Mycoplasma Fungi
TLR7	Single-stranded RNA Imidazoquinoline	Viruses Synthetic compounds
TLR8	Single-stranded RNA Imidazoquinoline	Viruses Synthetic compounds
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	Not determined	Not determined
TLR11	Profilin-like protein	Uropathogenic bacteria (<i>T.gondii</i>)

Table 2. Normal vaginal flora and microbes associated with genitourinary tract infection.

Type and sub-type	Normal vaginal flora	Microbes associated with genitourinary tract infection
Gram-negative aerobic bacteria	<i>Escherichia coli</i> <i>Proteus vulgaris</i> <i>Klebsiella</i> <i>Enterobactor</i>	<i>Escherichia coli</i> <i>Proteus vulgaris</i> <i>Klebsiella</i> <i>Enterobactor</i> <i>Acinetobactor calcoaceitus</i> <i>Pseudomonas aeroginosa</i> <i>Serratia</i> <i>Neisseria gonorrhoeae</i>
Gram-negative anaerobic bacteria	<i>Bacteroids fragilis</i> <i>Bacteroids urolyticus</i> <i>Pervotella</i> <i>Mobiluncus spp.</i>	<i>Bacteroids fragilis</i> <i>Bacteroids urolyticus</i> <i>Pervotella</i> <i>Mobiluncus spp.</i> <i>Porphyromonas</i> <i>Chlamydia trachomatis</i> <i>Gardnerella vaginalis</i>
Gram-positive aerobic bacteria	<i>Enterococcus faecalis</i>	<i>Enterococci</i> <i>Staphylococcus saprophyticus</i> <i>Staphylocococcus aureus</i>
Gram-positive anaerobic bacteria	<i>Streptococcus faecalis</i> <i>Staphylococcus epidermidis</i> <i>Lactobacillus acidophilus</i> <i>Clostridium</i>	<i>Peptostreptococcus</i> <i>Mycoplasma hominis</i>
Protozoa		<i>Trichomonas vaginalis</i>
Yeast		<i>Candida albicans</i> <i>Blastomyces</i> <i>Coccidioides immitis</i>