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Increased Expression of Pattern Recognition Receptors and Nitric Oxide Synthase in Patients with Endometriosis

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Abstract

Objective: Endometriosis is characterized by repeated inflammatory changes and serious adhesions, inducing innate and adaptive immune responses within the abdominal cavity. To assess these immune responses, we evaluated the levels of expression of Toll-like receptors (TLR)-1, -2, -4, -5, and -9; nucleotide-binding oligomerization domains (NOD)-1 and -2; interleukins-1 β , -6, -8, -10, and -12; interferon- γ ; tumor necrosis factor- α ; inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS); and immunoglobulins (lgs) in patients with endometriosis.

Methods: The levels of TLRs, NODs, cytokines, and NOS mRNAs in peritoneal effusions were assessed by real time reverse transcription-polymerase chain reaction; and IgG, IgA and IgM concentrations were measured by enzyme-linked immunosorbent assays (ELISA) in 40 patients with and 40 without endometriosis. Findings from the two groups were compared.

Results: We observed expression of all pattern recognition receptors (PRRs), cytokines, and NOS mRNAs and Igs in the effusion fluid of patients with and without endometriosis. The levels of TLR-2 and -9; NOD-1 and -2; iNOS and eNOS mRNAs and CA 125 were significantly higher in the endometriosis than in the non-endometriosis group (p<0.05 each). Moreover, PRR, cytokine, and NOS expression showed significant correlations (p<0.05).

Conclusions: PRRs, cytokines, and NOS, which act cooperatively in the innate immune response, are closely associated with endometriosis. Increased expression of TLR-2, TLR -9, NOD-1, NOD-2, and NOS mRNA in peritoneal fluid may be associated with endometriosis.

Key words: Endometriosis - Pattern Recognition Receptors, Toll-like receptors - Nitric oxide synthase, cytokine, Immunoglobulin

Introduction

Endometriosis is a disease related to chronic pelvic inflammation and associated pelvic pain that may be accompanied by, for example, dysmenorrhea, dyspareunia, infertility and menstrual irregularities. Although the pathogenesis of endometriosis has not been clearly defined, abnormal levels of immune system cells, including macrophages, dendritic cells and natural killer cells, have been observed within the abdominal cavities of patients with endometriosis. These cells, however, are unable to detect and eliminate ectopic endometrial cells. Moreover, immune system cells in the abdominal cavity were found to be dysfunctional (1). Complicated reactions may occur within the abdominal cavity, due to endometriosis-induced secretion or reactions of cytokines, chemokines, nitric oxide, immunoglobulins, and immune cells. Triggered immune reactions signify the host recognition of infectious agents, but, if pathogens are not swiftly recognized, immune reactions necessary to fight infections do not occur. Thus, recognition of infectious agents is regarded as one of the crucial processes in the host immune system.

Pattern recognition receptors (PRRs) recognize unique molecular characteristics of pathogens and induce appropriate immune responses. PRRs respond to distinct molecular motifs of pathogens, their sites of expression in microorganisms and signaling (2). Among the various types of PRRs in humans are Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The principal TLRs involved in endometriosis are TLR-4, present on cell surfaces, and TLR-3, present on lysosome/endosome membranes (3).

Increased concentrations of lipopolysaccharide (LPS) in the peritoneal cavity or endometriotic fluid can trigger pelvic inflammation and TLR-mediated endometriosis (4). TLRs are also activated by endogenous ligands, including heat shock protein, S100, fibronectin, fatty acid, oxidized LDL, neutrophil elastase and hyaluronan. TLRs stimulated by LPS or endogenous ligands and oxidative stress activate NF-kB, upregulating cytokine secretion as pro-inflammatory cascades (3, 5). As this process proceeds, the adaptive immune system becomes involved, along with the innate immune system. Although several studies have assessed the expression of PRRs, cytokines, NOS, and immunoglobulins separately in patients with endometriosis, no study to date has analyzed the relationships of these molecules by measuring all of them at the same time. We therefore analyzed the expression of PRRs, which are involved in inflammatory and immune responses; NOS, which are involved in the female reproductive process; and Igs, which are involved in the adaptive immune response, in patients with and without endometriosis. We also analyzed the relationships among these molecules in the peritoneal cavities of patients with and without endometriosis according to patient age, parity and serum CA125 concentration.

Subjects & Methods

Subjects

Intraperitoneal fluid samples were obtained

from 80 patients who visited the Department of Obstetrics and Gynecology at our hospital between June 2011 and July 2012. Of these, 40 were positive for endometriosis on laparoscopy, a finding confirmed during histological examination after surgery. All patients enrolled in this study were in the proliferative stage. Of the patients with endometriosis, 27 had stage 1, 7 had stage 2, and 6 had stage 3 endometriosis; none had stage 4. The remaining 40 patients had benign tumors, with no evidence of endometriosis, including 26 patients with myomas, 5 with dermoid cysts, 2 with hydrosalphix, 3 with paratubal cysts, 1 with a serous borderline ovarian tumor, and 3 with non-pathologic specificities. During laparoscopy, peritoneal fluid was collected aseptically from the Douglas pouch, taking care to avoid bleeding. Patients were excluded if they had inflammatory diseases or hormone producing conditions, including pregnancy; if peritoneal fluid was contaminated with blood; or if no peritoneal fluid could be obtained. The samples were centrifuged at 1800 x g for 10 min; the supernatants were stored at -80°C in 1.5 ml aliquots; and the cell pellets were stored at -80°C in 1.5 ml aliquots after adding RNase inhibitor. The study protocol was approved by the institutional review boards (IRBs) of Vincent's Hospital, The Catholic University of Korea and Kyung Hee University Hospital, and informed consent was obtained from each patient (VC11TISI0091, KMC IRB 1236-02).

Real-time reverse transcription-polymerase chain reactions

Total RNA was extracted from peritoneal fluid using RNA-Bee solution kits (Tel-Test, Friendswood, TX, USA), as described by the manufacturer. First-strand cDNA was synthesized by reverse transcription in 20 μ l of a reaction mixture containing 1 μ g of RNA, 1x reaction buffer, 1 mM dNTP, 5 µM random primers, 20 units RNase inhibitor, and 20 units AMV reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture was incubated at 42°C for 1 h, and the reaction was terminated by heating at 95°C for 5 min. Primers specific for Toll-like receptors (TLR)-1, -2, -4, -5, 6, and -9; nucleotide-binding oligomerization domains (NOD)-1 and -2; interleukins (IL)-1 β , -6, -8, -10, and -12; interferon- γ ; tumor necrosis factor-a; inducible nitric oxide synthase (NOS); and endothelial NOS are shown in Table 1. Real-time polymerase chain reactions (PCR) were performed using a Chromo4 Detector real-time system (Bio-Rad, Hercules, CA, USA) and the SsoFast EvaGreen supermix (Bio-Rad). Each 20-µl PCR reaction contained 2 μl of cDNA, 10 μl SsoFast EvaGreen supermix, 2 μl of each primer and 6 µl PCR grade water. The amplification protocols consisted of an initial denaturation

at 95°C for 30 sec, followed by 45 cycles of denaturation at 95°C for 5 sec and annealing and extension at 55°C to 64°C for 12 sec. The point at which expression of each cDNA crossed that of β -actin cDNA was applied to the formula, 2-(target gene- β actin), and the relative amounts of each cDNA were quantitated.

Enzyme-Linked Immunosorbent Assay (ELISA)

IgG, IgA, and IgM concentrations in the supernatants of peritoneal fluid were measured by ELISA. Briefly, 50 µl 1:100 goat anti-human IgG, IgA, and IgM in coating buffer (1.59 g Na₂CO₃+2.93 g NaHCO₃+5% NaN₃, pH 9.6) were placed into each well of a 96 well plate and incubated overnight at 4°C. The wells were washed 6 times and incubated with blocking antibody. To each well was added 50 µl of sample, and the plates were incubated at room temperature for 3 hours. The wells were washed 6 times, followed by incubation at room temperature with purified goat

Table I. Primers for real-time RT-PCR

anti-human IgG, IgA, or IgM conjugated to horseradish peroxidase in PBS/Tween/BSA solution. The plates were again washed 6 times and incubated with substrate solution (2, 2'-AZINO-Bis), and the optical absorbance of each well was measured at 450 nm (Bethyl Laboratories, Montgomery, TX).

Statistical analysis

The Kolmogorov-Smirnov test was used to assess normality and Levene's test was used to assess the equality of variances between groups. Between group differences in expression were determined using independent t-tests, with correlations assessed using Pearson correlation tests. Results according to endometriosis stage were analyzed using the Kruskal-Wallis test.

All statistical analyses were performed using SPSS version 13, with a p-value less than 0.05 considered statistically significant.

Name	Sequences	Annealing	Product size
	-	temperature	(bp)
TLR1	F:5'-CTATACACCAAGTTGTCAGC-3'	60	220
	R:5'-GTCTCCAACTCAGTAAGGTG-3'		
TLR2	F:5'-GCCAAAGTCTTGATTGATTGG-3'	64	347
	R:5'-TTGAAGTTCTCCAGCTCCTG-3'		
TLR4	F:5'-TGGATACGTTTCCTTATAAG-3'	56	507
	R:5'-GAAATGGAGGCACCCCTTC-3'		
TLR5	F:5'-CTAGCTCCTAATCCTGATG-3'	56	438
	R:5'-CCATGTGAAGTCTTTGCTGC-3'		
TLR6	F:5'-CCTCCCAGGATCAAGGTACTTG-3'	60	327
	R:5'-ATCAGGCCAGCCCTCTAACAC-3'		
TLR9	F:5'-CCCTCAACTTCACCTTGGATCT-3'	64	408
	R:5'-CCACATATGGCCCAGTGCA-3'		
IL-1β	F:5'-TGATGGCTTATTACAGTGGCAATG-3'	140	60
	R:5'-GTAGTGGTGGTCGGAGATTCG-3'		
IL-6	F:5'-GTGTTGCCTGCTGCCTTC-3'	60	194
	R:5'-AGTGCCTCTTTGCTGCTTTC-3'		
IL-8	F:5'-GACATACTCCAAACCTTTCCAC-3'	60	160
	R:5'-CTTCTCCACAACCCTCTGC-3'		
IL-10	F:5'-GAACCAAGACCCAGACATC-3'	60	137
	R:5'-CATTCTTCACCTGCTCCAC-3'		
IL-12p40	F:5'-TCGGCAGGTGGAGGTCAGC-3'	60	77
	R:5'-CGCAGAATGTCAGGGAGAAGTAGG-3'		
IFN-γ	F:5'-TGTGGAGACCATCAAGGAAGAC-3'	60	121
	R:5'-TGCTTTGCGTTGGACATTCAAG-3'		
TNF-a	F:5'-ATCTTCTCGAACCCCGAGTG-3'	60	51
	R:5'-GGGTTTGCTACAACATGGGC-3'		
iNOS	F:5'-TGGATGCAACCCCATTGTC-3'	60	59
	R:5'-CCCGCTGCCCAGTTT-3'		
eNOS	F:5'-CGGCATCACCAGGAAGAAGA-3'	67	60
	R:5'-CATGAGCGAGGCGGAGAT-3'		
β-actin	F:5'-GCGAGAAGATGACCCAGATC-3'	60	77
	R:5'-GGATAGCACAGCCTGGATAG-3'		

RT-PCR: real time-polymerase chain reaction; TLR: Toll-like receptor; NOD: nucleotide-binding oligomerization domain.

Results

Characteristics of Patients in the Endometriosis and Non-Endometriosis Groups

The 40 endometriosis patients included 18 nulliparas and 22 multiparas, of mean age 36.5 ± 8.8 years. The 40 non-endometriosis patients included 13 nulliparas and 27 multiparas, of mean age 40.6 ± 10.6 years. There were no between group differences in age, body mass index (BMI), fertility, or history of prior surgery (p>0.05 each). However, mean serum CA125 concentration was significantly higher in the endometriosis than in the non-endometriosis group (51.0±54.9 IU/ml vs 26.7±24.8 IU/ml, p<0.05).

Expression of TLR, NOD, IL, IFN- γ , TNF- α , iNOS and eNOS mRNAs in peritoneal fluid (Table 2)

We found that mRNAs encoding all PRRs, cytokines and NOS were present in the effusion fluid of patients in the endometriosis and non-endometriosis groups. The level of expression of each was higher in the endometriosis group, with the levels of expression of mRNAs encoding TLR-2 and -9; NOD-1 and -2; iNOS and eNOS being significantly higher in the endometriosis than in the non-endometriosis group (p<0.05).

Concentrations of Igs in effusion fluid

The concentrations of IgG (1759 ± 379 µg/ml vs 1670 ± 590 µg/ml) and IgA (875 ± 449 µg/ml vs 817 ± 359 µg/ml) were higher, whereas the concentration of IgM was lower (242 ± 128 µg/ml and 291 ± 151 µg/ml), in the endometriosis than in the non-endometriosis group. However, none of these differences was statistically significant (p>0.05 each).

Correlations of clinical manifestations with expression of PRRs, cytokines, NOS, and Igs (Table 3)

No significant correlation was observed between any clinical or demographic characteristic, including age, parity, or CA125 concentration, and the level of cytokine or NOS mRNA expression or Ig concentration, in the two patient groups (p>0.05 each).

Correlation of PRR, cytokine, and NOS mRNAs with Ig concentrations (Table 4)

Significant correlations were observed among the expression of mRNAs encoding PRRs, including TLRs-1, -2, -4, -5, 6, and -9; NODs-1 and -2; ILs-1 β , -6, -8, -10, and -12; IFN- γ ; TNF- α , and iNOS and eNOS in the two groups (p<0.05 each) (Table 4). In the endometriosis group, however, none of these mRNAs was correlated with the concentrations of IgG, IgM, and IgA (p>0.05 each).

Expression of TLR, NOD, NOS, and cytokine mRNAs according to stage of endometriosis

In analyzing the expression of TLR, NOD, NOS, and cytokine mRNAs according to the stage of endometriosis, we found that the levels of expression of TLR2, TLR4, and TLR5 increased significantly with increasing stage of endometriosis (p< 0.05 each) (Table 5). Significant differences were also observed when these patients were divided into two groups, those with Stage 1 and those with Stage 2 and higher (p< 0.05 each) (Table 6).

 Table 2. TLR, cytokine and NOS mRNA expression in the peritoneal fluid of patients with and without endometriosis.

	Endometrio	sis group	Non-endome	P value	
			group		
	Mean	SD	Mean	SD	
TLR1	0.678	1.824	0.107	0.389	0.074
TLR2	0.828	2.441	0.081	0.293	0.007
TLR4	0.004	0.016	0.000	0.001	0.175
TLR5	0.013	0.046	0.000	0.001	0.153
TLR6	6.046	19.816	0.739	3.115	0.133
TLR9	8.669	19.843	0.618	1.694	0.022
NOD1	1.132	3.851	0.027	0.077	0.014
NOD2	0.008	0.021	0.004	0.014	0.048
iNOS	0.131	0.330	0.011	0.016	0.036
eNOS	5.838	13.943	0.419	1.146	0.026
IL-1β	0.093	0.292	0.032	0.110	0.240
IL-6	0.090	0.271	0.126	0.683	0.773
IL-8	2.802	10.911	0.453	2.017	0.200
IL-10	0.056	0.103	0.078	0.249	0.632
IL-12	0.622	2.594	0.550	3.180	0.915
IFN-r	0.004	0.007	0.019	0.084	0.322
TNF-a	0.085	0.408	0.086	0.465	0.991

SD; standard deviation

TLR: Toll-like receptor; NOD: nucleotide-binding oligomerization domain; iNOS: inducible nitric oxide synthase ; eNOS: endothelial NOS; IL: interleukin; IFN-γ: interferon-γ; TNF-α: Tumor necrosis factor-α

Paranovi S Cafficients Pvalue Purson's cafficients Portants Age ILR1			Endometrie	osis group	Control group			
AgeTIRI0995.672.48.7.17TIRS066.710233.7.67TIRS066.766192.338TIRS066.766192.338TIRS.066.766220.318TIRS.067.044.277.1.145.410NOD12044.277.1.45.412LILP.108.577.4.65.220.242TILS.107.325.2.248.322TILS.107.325.2.48.322TILS.107.303.868.343NOD2.202.203.814.355TILS.107.303.868.343NOC3.229.203.101.900NOC3.229.203.101.901NOC3.229.203.101.901NOC3.229.105.211.909NOC3.209.905.121.909NOC3.209.933.916.949NOC3.209.933.916.949NOC1.006.973.911.912NOC2.923.913.921.924NOC3.926.933.916.924NOC4.939.948.933.948NOC5.211.224.948.940NOC4.937.946.933.948NOC5.214.946.948 <th></th> <th></th> <th>Pearson's Coefficients</th> <th>P value</th> <th>Pearson's coefficients</th> <th>P value</th>			Pearson's Coefficients	P value	Pearson's coefficients	P value		
ILI42068.7.02.35.2.37ILI44066.7.66.7.92.3.83ILI630.66.7.66.7.20.3.65ILI69.0.89.6.11.1.13.4.10NOD10.44.7.97.1.46.4.00NOD120.45.7.66.7.80.3.42IL-16.0.707.6.87.9.87.4.25IL-16.0.707.6.87.9.26.2.18IL-16.0.707.6.85.2.21.2.25IL-10.0.98.5.98.0.33.2.21IL-12.0.775.6.55.2.21.2.25IK-14.1.91.2.99.0.14.9.58NOD5.2.723.1.02.1.96.2.73NOD5.2.73.1.22.0.16.3.66NOD5.2.73.1.12.0.16.3.66NOD5.2.73.1.12.0.16.3.66NOD5.2.73.1.12.3.16.3.66NOD5.2.73.1.12.3.16.3.66NOD5.2.73.1.12.3.16.3.66NOD5.2.73.1.12.3.16.3.66NOD5.2.73.2.71.3.17.7.7IL41.1.02.3.08.3.77.4.16NOD1.0.62.7.38.2.01.3.38IL42.0.86.3.33.3.16.3.26NOD2.6.62.3.73.2.17.2.57IL44.1.08.3.33.1.08.4.26 </td <td>Age</td> <td>TLR1</td> <td>099</td> <td>.567</td> <td>248</td> <td>.171</td>	Age	TLR1	099	.567	248	.171		
1114 065 .019 23 .343 17185 066 .766 .192 .383 17186 048 .299 .2.40 .365 1149 .049 .611 .123 .410 NOD2 044 .277 .1.46 .410 NOD2 .045 .776 .635 .212 1.4.3 .103 .587 .2.48 .322 1.4.1 .098 .538 .033 .721 1.4.2 .477 .655 .2.10 .266 1.4.3 .107 .325 .2.48 .322 1.4.4 .107 .325 .2.48 .322 1.4.4 .107 .33 .052 .021 1.4.4 .107 .33 .042 .049 .166 .202 .223 .052 .041 .166 .203 .494 .345 .040 .167 .263 .275 .284<		TLR2	068	.710	263	.176		
ILKS -1056 269 -120 348 ILKS -048 229 -240 135 ILK9 0.09 611 -135 410 NOD1 -046 276 -238 142 IL-10 0.08 706 -238 142 IL-6 0.70 687 -486 371 425 IL-6 0.70 687 -486 372 122 IL-10 0.98 558 0.63 273 120 -196 267 IN-7 1.91 239 -0.04 .955 201 200 IN-7 1.91 239 -0.04 .956 273 120 -196 (aCG 2.00 195 -0.01 .900 196 -0.01 .900 IgA .339 0.32 .043 .401 .401 .401 .401 .401 .401 .401 .401 .401 .401 .401 .401		TLR4	095	.619	235	.247		
11.4% -1048 .0% -2.0% .146 11.4% .0489 .011 -1.35 .46 NCD2 -0.46 .277 .146 .410 NCD2 .046 .276 .282 .142 11-6 .070 .687 .288 .083 .212 11-4 .167 .325 .248 .122 .036 .021 .226 11-12 .075 .655 .210 .226 .044 .036 .278 11-12 .075 .655 .210 .226 .044 .036 .278 11-12 .075 .055 .210 .226 .042 .038 .042 .788 11-14 .122 .033 .066 .041 .036 .041 .046 .046 11-18 .019 .539 .182 .019 .041 .042 .048 .041 .042 .041 .042 .041 .042 .041		TLR5	056	.766	192	.338		
NOD1		TLR6	048	.789	260	.165		
NOD2 -045 .796 -280 .140 L1:1P -1.04 .377 145 .142 L1:4P -1.07 .657 .087 .082 L1:4 -1.07 .655 .020 .226 L1:4 -1.075 .655 .021 .226 L1:10 .098 .558 .063 .721 L1:2 075 .655 .202 .225 TNN-sa .172 .303 .056 .378 NOD5 .202 .223 .062 .799 NOS6 .202 .233 .062 .799 NO .288 .044 .316 .060 NO .288 .044 .316 .060 NO .283 .042 .339 .121 .240 NOD1 .063 .203 .461 .062 .233 .201 .286 NOD1 .063 .215 .248 .201 .285		ILK9 NOD1	.089	.611	153	.410		
NOL2 -040 570 -145 .142 II-16 .070 .687 485 .428 II-6 .070 .687 288 .125 II-10 .068 .565 .210 .265 II-11 .077 .455 .210 .265 II-12 .077 .455 .210 .265 II-13 .066 .221 .222 .062 .719 IIA5 .073 .102 .016 .040 .041 .943 IIA6 .202 .223 .062 .719 .933 .942 .949 IIA6 .021 .933 .942 .949 .949 .949 .949 .944 <		NODI	-:044	.797 706	140	.410		
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IL.8 167		IL-1p IL-6	104 070	.547	145 - 087	.412		
IL.10 .098 .538 .063 .221 IL.12 .075 .655 .210 .228 IRN-1 .172 .033 .056 .248 INN-1 .172 .033 .056 .248 NOS .273 .022 .223 .062 .793 Ig G .209 .195 .021 .933 Ig A .339 .032 .043 .844 Ig M .268 .094 .316 .060 Ig A .339 .322 .337 .151 .422 Ig A .239 151 .422 .337 .151 .422 Ig A .022 .337 151 .422 .337 151 .422 Ig A .012 .062 .733 211 .224 .234 .201 .234 .241 .241 .241 .241 .241 .241 .241 .241 .241 .241 .241 .24		IL-8	167	.325	248	.152		
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FN-r19129.014975TK-ra.172.30.065.748iNO5.273.102.196.267eNO8.202.223.062.799Ig G.209.95.021.903Ig A.339.042.044.944Ig M.268.094.946.946Ig A.339.042.946.946Ig A.209.693.182.946Ig M.208.693.197.315TR A.122.537.151.466TR B.062.738.201.288TR B.062.738.201.288NOD2.062.733.211.271IL 10.197.248.063.846NOD2.062.733.211.272IL 10.197.248.061.642IL 10.197.248.061.642IL 10.197.248.061.642IR A.168.278.040.819Ig A.111.242.346.343Ig A.113.361.141.442Ig A.146.460.345.149IR A.146.460.345.149Ig A.146.460.345.346Ig A.146.368.369.346Ig A.146.368.369.345Ig A.146.368 <td></td> <td>IL-12</td> <td>075</td> <td>.655</td> <td>210</td> <td>.226</td>		IL-12	075	.655	210	.226		
Parity1723030.05248NOS2022230.62719lg G2.091.95-0.21903lg A3.390.920.944.84lg M2.680.943.160.00TRI-1.095.99-1.973.15TRA-1.825.97-1.514.62TIRA-0.627.38-2.012.88TRA-0.627.38-2.012.88TRA-0.627.38-2.012.88TRA-0.627.38-2.012.88TRA-0.627.38-2.012.88TRA-0.627.38-2.012.88TRA-0.627.38-2.012.88TRA-1.054.81-1.083.46NOD1-0.637.250.288.76NOD2-0.627.33-1.912.72TRA-1.299.81-0.029.83TL20.388.77-1.912.72TNA2.022.44-0.816.24NOS2.112.24-0.816.24NOS2.112.24-1.823.94NOS2.112.24-1.823.94NOS2.112.24-1.823.94NOS2.112.24-1.823.94NOS2.112.24-1.823.94NOS2.112.24-1.923.94NOS2.11<		IFN- x	.191	.259	014	.935		
NNOS273102-196277NOS2022230.62779Ig G209195-021903Ig A3390.820.94844Ig M2.680.943.160.060ParityTIR-1095.39-182319TIR4-1225.37-1514.26TIR5-0766.97-1165.56TIR6-0627.38-0152.86NOD1-0637.250.288.66NOD2-0627.33-2112.71TI-161.25481-1085.61NOD2-0627.33-2112.71TI-17-1.161.972.48-0.92NOD2-0627.33-2112.72TI-161.972.48-0.928.65TI-171.972.48-0.928.65TI-161.972.48-0.928.65TI-171.972.48-0.923.66TI-161.972.48-0.923.65TI-171.912.723.66-0.93NCS1.162.73-0.408.99Ig A4.1654.00-1.194.91Ig A4.1654.00-1.194.92Ig A4.1654.063.45-1.44NCS1.165.74-3.082.92Ig A4.1654.063.45-1.44Ig A <td></td> <td>TNF-a</td> <td>.172</td> <td>.303</td> <td>.056</td> <td>.748</td>		TNF-a	.172	.303	.056	.748		
eNOS		iNOS	.273	.102	196	.267		
βg G		eNOS	.202	.223	.062	.719		
kg A		Ig G	.209	.195	021	.903		
ParityIg M.2.68.0.94.3.16.0.00ParityTLR1109.539182.319TLR2089.6.39197.315TLR4.122.537.151.462TLR5076.6.97.116.565TLR6.062.738201.288TLR9.036.843033.861NOD1063.725.028.876NOD2.062.733211.271IL-1β125.481108.541IL-6.009.961.077.666IL-8.168.333198.255IL-10.197.248032.833IL-12.399.566.314.642NNF-a.159.356035.840NOS.211.224182.302PIN-a.159.356035.840NOS.211.224182.302PIN-a.159.356.010.119.491Ig M.198.222.136.430Ig A.415.010.119.491Ig A.127.553.290.258Ig A.146.476.318.188Ig A.146.476.318.385Ig A.127.553.290.258Ig A.138.492.336.012Ig A.146.476		Ig A	.339	.032	.034	.844		
Parity TLR1 109 .539 182 .319 TLR2 089 .639 197 .315 TLR4 122 .537 151 .462 TLR5 .076 .697 .116 .565 TLR6 .062 .738 201 .288 TLR5 .062 .733 211 .271 IL79 .062 .733 211 .271 IL79 .062 .733 211 .271 IL79 .125 .481 .108 .541 IL46 .009 .961 .077 .666 IL78 .168 .333 198 .255 IL41 .168 .333 191 .272 IL70 .197 .248 .032 .883 IL42 .038 .827 191 .272 INF-q .159 .211 .224 .182 .302 INOS .218 </td <td></td> <td>Ig M</td> <td>.268</td> <td>.094</td> <td>.316</td> <td>.060</td>		Ig M	.268	.094	.316	.060		
TIR4 -1.089 6.69 -1.97 3.15 TIR5 -0.76 .697 -1.16 5.65 TIR6 -0.62 .738 201 2.88 TIR9 0.36 .423 .028 .876 NOD1 -0.63 .725 .028 .876 NOD2 -0.62 .733 .211 .271 IL-19 125 .481 108 .541 IL-46 .009 .961 .077 .666 IL-8 .168 .333 198 .255 IL-10 .197 .248 .032 .873 IL-12 .038 .827 191 .272 IN-7 .202 .244 .081 .662 NOS .111 .224 .181 .642 NN-7 .202 .244 .182 .302 eNOS .186 .278 .040 .819 ig G .415 .010 .119<	Parity	TLR1	109	.539	182	.319		
11.144 -1.12 3.57 -1.91 492 TLR5 -0.062 7.78 -2.01 288 TLR9 0.36 8.43 -0.03 8.61 NOD1 -0.63 7.25 0.28 8.76 NOD2 -0.62 7.73 -2.11 2.71 IL-1β -1.25 4.81 -1.08 5.44 IL-6 0.009 9.61 0.077 6.66 IL-8 1.68 3.33 198 2.25 IL-10 1.977 2.48 -0.32 8.85 IL-12 0.38 8.27 191 2.22 FN-ra 1.259 3.56 035 8.40 iNOS 2.111 2.24 182 .30 eNOS 1.86 2.78 040 8.19 iRG 2.90 0.78 149 .349 iRG 1.19 4.91 .345 .148 ig M 1.98 2.22 <		TLR2	089	.639	19/	.315		
LLKS 0/6 .09 -1.16 .080 TLR6 062 .788 201 .288 TLR9 .036 .843 .003 .861 NOD1 063 .725 .028 .876 NOD2 062 .733 211 .271 L-1β 125 .481 108 .541 L-6 009 .961 077 .666 L.8 168 333 198 255 L.10 197 .248 032 853 L-12 038 827 191 224 INF-α 159 356 035 840 NOS 211 224 182 302 eNOS 186 278 040 819 Ig A 115 010 119 491 Ig A 159 366 331 224 Ig A 165 4		ILR4	122	.537	151	.462		
LLNB 002 .7.35 2.01 .2.85 TLR9 .036 .843 033 .861 NOD1 063 .725 .028 .876 NOD2 062 .733 211 271 IL-1β 125 .481 108 51 IL-10 197 .248 032 85 IL-10 197 .248 032 85 IL-10 197 248 032 85 IL-10 197 248 032 85 IL-10 197 248 032 85 IL-12 038 827 191 272 IFN-r 202 244 182 302 iNOS 211 224 182 302 iNOS 211 224 182 312 ig A 115 010 119 313 244 Ig A		TLR5	076	.697	116	200.		
Info 1000 1000 1000 1000 1000 NOD1 -062 773 -211 271 IL-10 -1125 481 -108 541 IL-6 0.09 961 0.077 666 IL-10 1.97 248 -0.02 2853 IL-10 1.97 248 -0.02 2853 IL-12 0.38 827 -191 272 FN-r 2.02 2.24 -0.81 642 TNF-a 1.159 356 -0.05 840 iNOS 2.11 2.24 -182 302 eNOS 1.86 2.78 -0.40 819 ig G 2.90 0.78 1.49 385 ig A 4.15 0.10 -119 491 ig A 1.16 5.31 2.92 1.36 ig A 1.16 3.11 2.24 1.83 ig A 1.16 5.11 2.8		TLR0	062	./ 30 8/3	201	.200 861		
NOD2		NOD1	- 063	.045	055	.001		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		NOD2	- 062	733	- 211	.070		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		IL-1B	125	.481	108	.541		
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		IL-6	.009	.961	.077	.666		
II-10 .197 .248 032 .853 II.12 .038 .827 .191 .272 FN-r .202 .244 .081 .642 FN-a .159 .356 .035 .840 iNOS .211 .224 .182 .302 eNOS .186 .278 .040 .819 ig G .290 .078 .149 .335 ig A .415 .010 .119 .491 ig M .198 .232 .136 .430 TLR2 .165 .440 .311 .224 TLR4 .144 .511 .288 .279 TLR5 .127 .553 .290 .288 TLR6 .146 .476 .318 .185 TLR5 .127 .553 .290 .288 TLR6 .146 .476 .318 .185 TLR5 .127 .306 .247		IL-8	.168	.333	198	.255		
IL-12 .038 .827 .1-191 .272 IFN-r .202 .244 .081 .642 TNF-a .159 .356 .035 .840 iNOS .211 .224 .182 .302 eNOS .186 .278 .040 .819 Ig G .290 .078 .149 .385 Ig A .415 .010 .119 .491 Ig M .198 .232 .136 .430 CA125 TLR1 .089 .660 .345 .148 TLR2 .165 .440 .311 .224 TLR3 .127 .553 .290 .288 TLR6 .146 .476 .318 .325 NOD1 .102 .612 .237 .300 NOD2 .116 .574 .308 .214 IL-19 .138 .492 .566 .028 .214 IL-46 .368		IL-10	.197	.248	032	.853		
IFN-r 202 244 -081 642 TNF-α .159 .356 035 .840 iNOS .211 .224 .182 .302 eNOS .186 .278 040 .819 Ig G .290 .078 .149 .385 Ig A .415 .010 .119 .491 Ig M .198 .232 .136 .430 TLR1 .089 .660 .345 .148 TLR2 .165 .440 .311 .224 TLR4 .144 .511 .288 .279 TLR5 .127 .553 .290 .288 TLR9 .372 .056 .298 .216 NOD1 .102 .612 .237 .300 NOD2 .116 .574 .308 .214 IL-6 .368 .059 .198 .389 IL-6 .368 .059 .198 <t< td=""><td></td><td>IL-12</td><td>.038</td><td>.827</td><td>191</td><td>.272</td></t<>		IL-12	.038	.827	191	.272		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		IFN- x	.202	.244	081	.642		
iNOS 211 224 -182 302 eNOS .186 .278 .040 .819 Ig G .290 .078 .149 .385 Ig A .415 .010 .119 .491 Ig M .198 .232 .136 .430 TLR1 .089 .660 .345 .148 TLR2 .165 .440 .311 .224 TLR4 .144 .511 .288 .279 TLR5 .127 .553 .290 .258 TLR6 .146 .476 .318 .185 TLR6 .127 .553 .290 .258 TLR5 .127 .536 .012 .237 .300 NOD1 .102 .612 .237 .300 .001 IL-6 .368 .059 .198 .389 .127 .565 IL-8 .104 .551 030 .894 .172 <t< td=""><td></td><td>TNF-a</td><td>.159</td><td>.356</td><td>035</td><td>.840</td></t<>		TNF-a	.159	.356	035	.840		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		iNOS	.211	.224	182	.302		
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		eNOS	.186	.278	040	.819		
Ig A.415.010119.491Ig M.198.232.136.430CA125TLR1.089.660.345.148TLR2.165.440.311.224TLR4.144.511.288.279TLR5.127.553.290.258TLR6.146.476.318.185TLR9.372.056.298.216NOD1.102.612.237.300NOD2.116.574.308.214L-1β.138.492.536.012L-6.368.059.198.389L-8.104.598.302.172JL-10.113.561114.615IL-12.149.440.287.195JFN-r.126.515.030.898JRG.067.719.003.988Jg G.067.719.003.988Jg A.213.270.551.030		lg G	.290	.078	.149	.385		
Ig M.198.252.136.430CA125TLR1.089.660.345.148TLR2.165.440.311.224TLR4.144.511.288.279TLR5.127.553.290.258TLR6.146.476.318.185TLR9.372.056.298.216NOD1.102.612.237.300NOD2.116.574.308.214L-16.368.059.198.389IL-8.104.598.302.172IFN-r.126.515.030.894TNF-q.012.953.127.555iNOS.169.390.310.171eNOS.067.719.003.988Ig G.067.719.003.988Ig A.213.250.047.811		lg A	.415	.010	119	.491		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	CA125	Ig M TI P1	.198	.232	.136	.430		
TIRE1.00.440.11.224TLR4.144.511.288.279TLR5.127.553.290.258TLR6.146.476.318.185TLR9.372.056.298.216NOD1.102.612.237.300NOD2.116.574.308.214IL-1β.138.492.536.012IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561.114.615IL-12.149.440.287.195IFN-x.126.515.030.894TNF-a.012.953.127.565iNOS.169.390.310.171eNOS.007.719.003.988Ig G.067.719.003.988Ig A.423.018.242.266Ic M.213.250.007.811	CAI25		.009	.000	.343	.140		
TLR.144.511.125.127TLR5.127.553.290.258TLR6.146.476.318.185TLR9.372.056.298.216NOD1.102.612.237.300NOD2.116.574.308.214IL-1β.138.492.536.012IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561114.615IL-12.149.440.287.195IFN-r.126.515.030.894TNF-a.012.953.127.565iNOS.169.390.310.171eNOS.067.719.003.988Ig G.067.719.003.988Ig A.213.250.047.818		TI R4	.105	.440	.511	.224		
TLR61.46476.101.851.85TLR9.372.056.298.216NOD1.102.612.237.300NOD2.116.574.308.214IL-1 β .138.492.536.012IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561114.615IL-12.149.440.287.195FN-x.126.515030.894TNF- α .012.953.127.565iNOS.169.390.310.171eNOS.011.957.119.588Ig G.067.719.003.988Ig A.423.018.242.266Ix M.213.250.047.831		TLR5	127	.511	.200	.279		
TLR9.772.056.298.216NOD1.102.612.237.300NOD2.116.574.308.214IL-1β.138.492.536.012IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561114.615IL-12.149.440.287.195IFN-r.126.515030.894TNF-α.012.953127.565iNOS.169.390.310.171eNOS.067.719.003.988Ig G.067.719.003.988Ig A.213.250.047.811		TLR6	.146	.476	.318	.185		
NOD1.102.612.237.300NOD2.116.574.308.214IL-1β.138.492.536.012IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561.114.615IL-12.149.440.287.195IFN-r.126.515030.894TNF-α.012.953127.565iNOS.169.390.310.171eNOS.017.957119.588Ig G.067.719003.988Ig A.423.018.242.266Ia M.213.250.047.831		TLR9	.372	.056	.298	.216		
NOD2.116.574.308.214 IL -1β.138.492.536.012IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561114.615IL-12.149.440.287.195FN-r.126.515030.894TNF-α.012.953.127.565iNOS.169.390.310.171eNOS.017.957.119.588Ig G.067.719.003.988Ig A.423.018.242.266Ia M.213.250.047.831		NOD1	.102	.612	.237	.300		
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		NOD2	.116	.574	.308	.214		
IL-6.368.059.198.389IL-8.104.598.302.172IL-10.113.561114.615IL-12.149.440.287.195IFN-x.126.515030.894TNF-a012.953127.565iNOS.169.390.310.171eNOS011.957119.588Ig G.067.719003.988Ig A.423.018.242.266Ia M.213.250.047.831		IL-1β	.138	.492	.536	.012		
IL-8.104.598.302.172IL-10.113.561114.615IL-12.149.440.287.195IFN-x.126.515030.894TNF-a012.953127.565iNOS.169.390.310.171eNOS011.957119.588Ig G.067.719003.988Ig A.423.018.242.266Ia M.213.250.047.831		IL-6	.368	.059	.198	.389		
IL-10.113.561114.615IL-12.149.440.287.195IFN-x.126.515030.894TNF-a012.953127.565iNOS.169.390.310.171eNOS011.957119.588Ig G.067.719003.988Ig A.423.018.242.266Ia M.213.250.047.831		IL-8	.104	.598	.302	.172		
IL-12.149.440.287.195IFN- x .126.515030.894TNF- α 012.953127.565iNOS.169.390.310.171eNOS011.957119.588Ig G.067.719003.988Ig A.423.018.242.266Ia M.213.250.047.831		IL-10	.113	.561	114	.615		
IFN-r.126.515030.894TNF- α 012.953127.565iNOS.169.390.310.171eNOS011.957119.588Ig G.067.719003.988Ig A.423.018.242.266Ia M.213.250.047.831		IL-12	.149	.440	.287	.195		
TNF-α 012 .953 127 .565 iNOS .169 .390 .310 .171 eNOS 011 .957 119 .588 Ig G .067 .719 003 .988 Ig A .423 .018 .242 .266 Im M .213 .250 .047 .831		IFN-r	.126	.515	030	.894		
INOS .169 .390 .310 .171 eNOS 011 .957 119 .588 Ig G .067 .719 003 .988 Ig A .423 .018 .242 .266 Ig M .213 .250 .047 .831		TNF-a	012	.953	127	.565		
encos 011 .957 119 .588 Ig G .067 .719 003 .988 Ig A .423 .018 .242 .266 Ig M 213 250 047 831		INUS	.169	.390	.310	.171		
Ig A .423 .018 .242 .266		enos La C	011	.957	119	.588		
18 M 213 250 047 831		Ig G Ig A	.067	./19	003	.988		
		Ig M	.425 213	.018	.242 047	.200 831		

TLR: Toll-like receptor; NOD: nucleotide-binding oligomerization domain; iNOS: inducible nitric oxide synthase ; eNOS: endothelial NOS; IL: interleukin; IFN-x: interferon-y; TNF-a: Tumor necrosis factor-a

	TLR1	TLR2	TLR4	TLR5	TLR6	TLR9	NOD1	NOD2	IL-1β	IL-6	IL-8	IL-10	IL-12	IFN-γ	TNF-α	iNOS	eNOS	IgG	IgA	IgM
TLR1	1	.979**	.942**	.947**	.979**	.822**	.962**	.922**	.860**	.855**	.043	.419**	.012	.537**	.602**	.166	.878**	065	.098	.015
TLR2	.979**	1	.979**	.987**	.997**	.781**	.994**	.905**	.770**	.832**	.019	.419**	.012	.537**	.602**	.166	.878**	065	.098	.015
TLR4	.942**	.979**	1	.997**	.985**	.680**	.981**	.862**	.731**	.752**	007	.405**	009	.377**	.429**	.013	.691**	099	.022	.022
TLR5	.947**	.987**	.997**	1	.989**	.704**	.991**	.864**	.733**	.770**	011	.408**	010	.375**	.422**	001	.695**	121	.040	.044
TLR6	.979**	.997**	.985**	.989**	1	.755**	.989**	.921**	.769**	.809**	.026	.420**	.007	.489**	.467**	.119	.768**	081	.076	.042
TLR9	.822**	.781**	.680**	.704**	.755**	1	.778**	.671**	.812**	.988**	.013	.343**	020	.353**	.538**	.024	.830**	069	.250*	.124
NOD1	.962**	.994**	.981**	.991**	.989**	.778**	1	.863**	.764**	.829**	012	.417**	009	.382**	.446**	011	.735**	098	.090	.058
NOD2	.922**	.905**	.862**	.864**	.921**	.671**	.863**	1	.672**	.706**	.137	.400**	.054	.774**	.417**	.476**	.776**	050	.079	.048
IL-1β	.860**	.770**	.731**	.733**	.769**	.812**	.764**	.672**	1	.824**	004	.337**	016	.344**	.901**	.006	.949**	.008	.179	032
IL-6	.855**	.832**	.752**	.770**	.809**	.988**	.829**	.706**	.824**	1	.014	.364**	017	.352**	.529**	.007	.823**	079	.218	.149
IL-8	.043	.019	007	011	.026	.013	012	.137	004	.014	1	.133	.482**	.044	034	.336**	030	063	098	052
IL-10	.419**	.417**	.405**	.408**	.420**	.343**	.417**	.400**	.337**	.364**	.133	1	.085	.949**	.919**	.027	.910**	067	062	008
IL-12	.012	.000	009	010	.007	020	009	.054	016	017	.482**	.085	1	.038	.013	.159	.016	.099	016	137
IFNy	.537**	.457**	.377**	.375**	.489**	.353**	.382**	.774**	.344**	.352**	.044	.949**	.038	1	.906**	.275*	.897**	031	018	.007
TNFa	.602**	.456**	.429**	.422**	.467**	.538**	.446**	.417**	.901**	.529**	034	.919**	.013	.906**	1	.012	.993**	041	060	.036
iNOS	.166	.079	.013	001	.119	.024	011	.476**	.006	.007	.336**	.027	.159	.275*	.012	1	.024	.065	041	001
eNOS	.878**	.768**	.691**	.695**	.768**	.830**	.735**	.776**	.949**	.823**	030	.910**	.016	.897**	.993**	.024	1	054	063	.059
IgG	065	087	099	121	081	069	098	050	.008	079	063	067	.099	031	041	.065	054	1	.384**	122
IgA	.098	.112	.022	.040	.076	.250*	.090	.079	.179	.218	098	062	016	018	060	041	063	.384**	1	073
IgM	.015	.042	.022	.044	.042	.124	.058	.048	032	.149	052	008	137	.007	.036	001	.059	122	073	1

Table 4. Correlations between IgG, IgA, and IgM concentrations and mRNAs encoding TLRs-1, -2, -4, -5, -6, and -9; NODs-1 and -2; ILs-1 β , -6, -8, -10, and -12; IFN- γ ; and TNF- α in endometriosis group

*p<0.05,** p<0.01. TLR: Toll-like receptor; NOD: nucleotide-binding oligomerization domain; iNOS: inducible nitric oxide synthase ; eNOS: endothelial NOS; IL: interleukin; IFN-x: interferon-γ; TNF-α: Tumor necrosis factor-α

	Number	Stage	Mean	SD	P value
TLR1	25	1	0.539	1.988	0.189
	6	2	1.037	1.686	
	5	3	0.941	1.204	
TLR2	23	1	0.679	2.686	.016
	5	2	1.518	2.193	
	4	3	0.818	1.136	
TLR4	21	1	0.004	0.019	.003
	5	2	0.007	0.012	
	4	3	0.002	0.001	
TLR5	22	1	0.012	0.052	.032
	5	2	0.019	0.036	
	4	3	0.008	0.013	
TLR6	23	1	5.438	22.677	.115
	6	2	9.196	16.527	
	5	3	5.061	7.192	
	24	1	4.747	14.425	.191
TLR9	6	2	19.807	34.838	
	5	3	14.129	17.826	
NOD1	25	1	0.982	4.286	.096
	6	2	1.791	3.441	
	5	3	1.091	1.987	
NOD2	24	1	0.007	0.024	.191
	6	2	0.012	0.016	
	5	3	0.009	0.011	
IL-1β	25	1	0.072	0.261	.561
	6	2	0.203	0.347	

Table 5. Expression of TLR, NOD, NOS, and cytokines according to stage of endometriosis

	5	3	0.341	0.557	
IL-6	25	1	3.135	10.364	.191
	6	2	14.708	25.143	
	5	3	8.706	9.613	
IL-8	26	1	0.100	0.340	.510
	6	2	0.132	0.165	
	5	3	0.013	0.015	
IL-10	27	1	0.113	0.318	.075
	6	2	0.041	0.065	
	5	3	0.025	0.032	
IL-12	27	1	3.739	12.885	.195
	6	2	0.785	0.955	
	5	3	0.160	0.165	
IFN- γ	26	1	0.059	0.123	.196
	6	2	0.041	0.025	
	5	3	0.060	0.035	
TNF-α	27	1	0.718	3.041	.087
	6	2	0.161	0.254	
	5	3	0.658	1.293	
iNOS	26	1	0.003	0.008	.061
	6	2	0.006	0.007	
	5	3	0.005	0.006	
eNOS	27	1	0.109	0.484	.121
	6	2	0.021	0.027	
	5	3	0.030	0.042	
	27	1	1739429.259	431206.009	.447
IgG	7	2	1695337.143	148262.886	
	6	3	1922398.333	297959.256	
IgA	27	1	816538.519	436065.112	.423
0	7	2	979875.714	382088.450	
	6	3	1017103.333	596140.403	
IgM	27	1	222407.778	107831.864	.220
2	7	2	323938.571	157043.179	
	6	3	237848.333	166191.940	

TLR: Toll-like receptor; NOD: nucleotide-binding oligomerization domain; iNOS: inducible nitric oxide synthase; eNOS: endothelial NOS; IL: interleukin; IFN-Y: interferon-y; TNF-a: Tumor necrosis factor-a

Table 6. Expression of TLR, NOD, NOS, and cytokines in patients with endometriosis stage 1 and with stage 2 or higher

	Stage 1		Stage 2 or more	Stage 2 or more			
	Mean	SD	Mean	SD			
TLR1	0.539	1.988	0.994	1.416	.080		
TLR2	0.679	2.686	1.207	1.739	.004		
TLR4	0.004	0.019	0.014	0.009	.001		
TLR5	0.012	0.052	0.014	0.028	.008		
TLR6	5.438	22.677	7.317	12.725	.038		
TLR9	4.747	14.425	17.226	27.253	.078		
NOD1	0.982	4.286	1.473	2.763	.035		
NOD2	0.007	0.024	0.011	0.013	.078		
iNOS	0.072	0.261	0.266	0.435	.018		
eNOS	3.135	10.364	11.980	19.049	.041		
IL-1β	0.100	0.340	0.078	0.132	.523		
IL-6	0.113	0.318	0.034	0.051	.095		
IL-8	3.739	12.885	0.501	0.757	.339		
IL-10	0.059	0.123	0.050	0.030	.028		
IL-12	0.718	3.041	0.386	0.877	.076		
IFN-x	0.003	0.008	0.006	0.006	.075		
TNF-a	0.109	0.484	0.025	0.033	.027		

TLR: Toll-like receptor; NOD: nucleotide-binding oligomerization domain; iNOS: inducible nitric oxide synthase ; eNOS: endothelial NOS; IL: interleukin; IFN-γ: interferon-γ; TNF-α: Tumor necrosis factor-α

Discussion

Endometriosis is a complex inflammatory disease of the pelvis, characterized by disparate morphological, histological and biochemical properties. We evaluated endometriosis immunologically by analyzing the expression of PRRs, cytokines, nitric oxide, and immunoglobulins in the peritoneal cavity and determining whether expression of any of these molecules was correlated with the clinical characteristics of endometriosis.

The inability of the immune system to promptly recognize invading pathogens can lead to various diseases since the host immune response against these pathogens may not occur or may be inappropriate. Thus, recognizing pathogens during the initial stage of disease is crucial. This process is initiated by PRRs, a group of molecules distributed in the extracellular, membrane, and cytoplasmic compartments. Humans have various PRRs, including TLRs and NOD-like receptors (NLRs), which differ by location. For example, TLRs-1, -2, -4, -5, -6 and -10 are present on the cell surface; TLRs-3, -7, -8, and -9 are located on lysosome/endosome membranes; and NODs-1 and -2 are present in the cytoplasm (2,6-7). Pathogens that escape extracellular or membrane detection systems may be recognized by PRRs in the cytoplasm or on lysosome/endosome membranes (8).

We found that patients with endometriosis expressed mRNAs encoding TLRs-1, -2, -4, -5, 6, and -9 and NODs-1 and -2, all PRRs associated with immunity against infection. Moreover, the levels of expression of mRNAs encoding TLRs-2 and -9 and NODs-1 and -2 were significantly higher in patients with than without endometriosis. Up-regulation of PRR expression and accelerated endometrial proliferation can result in tumor formation (9). Human heat-shock protein 70 has been reported to induce pelvic inflammation, involving the TLR-3 and TLR-4-mediated growth of endometrial cells (5, 10), with TLR-4 having a significant role in innate immune reactions to bacterial endotoxin in patients with endometriosis (4). Moreover, a TLR-4 polymorphism associated with hypo-responsiveness of the receptor may result in peritoneal inflammation. Thus, endometrial cells tend to adhere to the peritoneum, a condition that may induce the initiation of endometriosis (11). We found that PRRs other than TLR-3 and TLR-4 are involved in immune reactions in patients with endometriosis of the peritoneal cavity.

NO reacts to homeostatic and pathologic stimuli and is secreted by neurons, endothelial cells, platelets and neutrophils. NO also plays a crucial role in female reproductive processes, including ovulation, menstruation, implantation, pregnancy maintenance, and labor and delivery (12). A higher level of activated peritoneal macrophages has been observed in women with endometriosis compared to those without the condition, with iNOS expression and NO production increased due to the activation of peritoneal macrophages (13). The participation of peritoneal macrophages in antimicrobial and antitumor activities increases as NO concentration increases. Furthermore, increased NO concentrations can change pro-inflammatory and peritoneal immune defense reactions and may be involved in the pathogenesis of endometriosis (14). Of the three NO-synthase isoenzymes, eNOS and nNOS are constitutively expressed, whereas iNOS, while not normally present in macrophages, can be upregulated depending on the stimulus. The latter enzyme is also involved in vasodilation and the destruction of pathogens such as bacteria (15). Although the level of NOS is dependent on endometrial phase, association with infertility, position in the menstrual cycle, and types of cells and tissues, we found that the levels of expression of iNOS and eNOS were higher in the endometriosis than the non-endometriosis group (16, 17).

Cytokines, a group of glycoproteins that participate in modulating inflammatory and immune reactions in many diseases, were recently found to be involved in endometriosis in humans and experimental animals. Although most studies have reported that the secretion of cytokines increases during and after the process of endometriosis, other studies have reported that cytokine secretion decreases or does not change significantly.

We observed no differences in the levels of expression of ILs-1 β , -6, -8, -10, and -12, INF- γ , and between TNF-a our endometriosis and non-endometriosis groups. In contrast, other studies have reported higher expression of ILs-1ß, -6, -8, and -15, TNF-a, monocyte chemotactic protein-1 (MCP-1), eotaxin, regulated upon activation normal T-cell expressed and secreted (RANTES), and intercellular adhesion molecule-1 (ICAM-1), in patients with than without endometriosis (18-21). Several other studies, however, have found that the levels of expression of ILs-1ß, -10, -12, and -18, TNF-a, RANTES, VEGF, PDGF, sFas and sFasL, and vascular cell adhesion molecule-1 (VCAM-1) were similar, or in some cases lower, in patients with than without endometriosis (18, 22-24). Differences among study results may be due to the severity of endometriosis; polymorphisms in the genes encoding these molecules; types of samples, including endometriosis tissue, peritoneal fluid, or serum; the nature of the control group; or the use of medications by patients with endometriosis. Although none of our patients showed evidence of peritonitis or other inflammation before surgery, the ab-

dominal cavities of all 80 patients were positive for IgG, IgA, and IgM secretion. The concentrations of IgG and IgA were higher, and the concentration of IgM was lower, in our endometriosis group, but none of these differences was statistically significant. Although B cells in the peritoneal cavity cannot produce antibodies in a sterile environment, without infection or external stimuli, immunoglobulins are spontaneously produced by B-1, not B-2, cells in the peritoneal cavity even in the absence of exogenous infection (25, 26). B-1 cells express the pan T-cell surface glycoprotein, CD5, and are localized in the peritoneal and pleural cavities, with few, if any, found in the spleen (27, 28). Although we did not separate B-1 from B-2 cells, the antibodies in the peritoneal cavity likely resulted from the immune responses of B-1 cells.

When B cells differentiate into plasma cells, the initial immunoglobulins produced are IgM and IgD, with IgG, IgA, and/or IgE produced following a process called "class-switching" (27). Thus, various antibodies can be present in the peritoneal cavity in the absence of any exogenous infection. We focused on three classes of antibody: IgG, which is primarily involved in chronic inflammation and auto-immune reactions; IgA, which is primarily involved in mucosal immunity; and IgM, which is primarily involved in acute inflammation. We excluded IgD and IgE, which are present at much lower concentrations. Previous findings showing that the concentration of a specific IgG autoantibody was increased in the peritoneal fluid of patients with endometriosis and that endometrial glandular epithelial staining for both IgG and IgA was significantly increased, suggests that endometriosis may be an autoimmune disease (29, 30). Moreover, the increase in IgG concentration suggests that these patients may have had a precursor condition of endometriosis requiring treatment (31).

We found that inflammatory, innate, and adaptive immune reactions in the peritoneal cavity were integrated, with significant correlations among mRNAs encoding PRRs such as TLRs-1, -2, -4, -5, -6, and -9, and NODs-1 and -2; cytokines such as ILs-1 β , -6, -8, -10, and -12, INF- γ , and TNF- α ; and iNOS and eNOS. These PRRs, cytokines, and NOS are induced by external stimuli, antigens, and pathogens, via distinct pathways; following which they stimulate and facilitate the production of other signaling molecules and/or act synergistically. The manifestations and correlations of PRRs, cytokines, and NOS may be involved in the pathogenesis of endometriosis and in endometriosis-induced immune reactions in the peritoneal cavity.

Our study had several limitations. For ethical reasons, our control group consisted of patients with lesions in the peritoneal cavity, rather than disease free normal subjects. Although none of the control patients had infections in the peritoneal cavity, the peritoneal lesions may have induced immune reactions. Second, we measured the levels of expression of mRNA instead of protein, since some proteins were not expressed. Third, the response of tissues other than the endometrium was not examined since the study samples consisted only of peritoneal fluid and there have been no comparative studies of serum and endometrial tissue.

We have shown here that mRNAs encoding various PRRs, cytokines, and NOS, as well as IgG, IgA, and IgM, were expressed in the peritoneal cavities of patients with endometriosis, with this expression due to diverse inflammatory and immune reactions. The expression of several PRRs and NOS increased significantly, suggesting that the cooperative interaction of PRRs, cytokines, and NOS in innate immune responses in the peritoneal fluid may be associated with endometriosis.

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Competing Interests

The authors have declared that no competing interest exists.

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