Reproductive Sciences
2016, Vol. 23(1) 124-133

DOI: 10.1177/1933719115597761
rs.sagepub.com
SAGE

Cytokine Profiling in the Eutopic Endometrium of Adenomyosis During the Implantation Window After Ovarian Stimulation

N. Zhihong, MD[1], F. Yun, MD[1], Z. Pinggui, MD[1], Z. Sulian, MD[1], and Aijun Zhang, MD[1]

Abstract

In this study, we aimed to clarify the inflammatory cytokine profile of endometrium in patients with adenomyosis during the implantation window after ovarian stimulation. Eighteen patients with adenomyosis and 24 control patients undergoing in vitro fertilization treatment were included in this prospective case-control study. Regular gonadotropin-releasing hormone antagonist protocol was used for ovarian stimulation. Endometrial samples were obtained 7 days after human chorionic gonadotropin (hCG) injection (hCG + 7). Cytokine levels in endometrium secretions from women with and without adenomyosis were assayed by multiplex immunoassay, levels of interleukin (IL) 6 (25.9 ± 6.6 vs 12.4 ± 3.4 pg/mL; $P = .001$), IL-10 (10.4 ± 2.9 vs 15.6 ± 4.2 pg/mL; $P = .001$), IL-17 (11.9 ± 3.0 vs 14.2 ± 3.9 pg/mL; $P = .046$), interferon-γ (11.7 ± 3.5 vs 8.0 ± 3.4 pg/mL; $P = .001$), and monocyte chemoattractant protein-1 (MCP-1; 37.1 ± 6.5 vs 16.4 ± 3.2 pg/mL; $P = .001$) were significantly different between patients with adenomyosis and control groups, respectively. Immunohistochemistry and quantitative real-time polymerase chain reaction showed that CD-68+, IL-6, and MCP-1 expression were higher and IL-10 was lower in adenomyosis endometrium epithelia compared to controls. In conclusion, within the implantation window of ovarian stimulation cycles, macrophages, IL-6, IL-10, and MCP-1 are expressed differently in the endometrium of women with adenomyosis, which may correlate with compromised endometrium receptivity. We postulated that cytokines of endometrial secretions expressed differently in patients with adenomyosis may contribute to impaired endometrium receptivity in these patients.

Keywords

adenomyosis, cytokines, endometrium, ovarian stimulation

Introduction

Adenomyosis is a benign uterine disorder characterized by the presence of heterotopic endometrial glands, stroma in the myometrium, and reactive hyperplasia of the surrounding smooth muscle cells of the myometrium.[1] The etiology and pathologic mechanisms responsible for adenomyosis have yet to be elucidated. The incidence of newly diagnosed adenomyosis in infertile women is likely to increase as more women are delaying their first pregnancy into their 30s and 40s.[2]

To date, the effect of adenomyosis on in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment outcome is unclear due to only a limited number of small observational studies reporting either no effect or a poorer outcome associated with the presence of adenomyosis.[3-5] It has been postulated that the presence of abnormal vascularity and heterotopic glands in the myometrium of patients with adenomyosis may be a source of cytokines and immunological factors that can have a negative impact on endometrial receptivity and placentation.[6] There is also accumulating evidence that shows that adenomyosis has an effect on endometrial cell antigen expression, cytokine production, and production of oxygen-free radicals, all of which are suggestive of the disruption of endometrial receptivity.[7,8] In recent years, a novel means of assessing intrauterine secretion milieu by multiplex immunoassay has been developed[9,10]; this technique has potential value for investigating intrauterine environment during the implantation window. In women with adenomyosis, some immunological cytokines expression within endometrial has been found to be significantly altered, such as interleukin (IL) 6, IL-11, and IL-10.[11] Furthermore, some studies have shown that different cytokine profiles in ovarian-stimulated cycles are associated with the likelihood of conceiving. Boomsma et al reported significant associations between monocyte chemoattractant protein-1 (MCP-1), interferon (IFN) γ-inducible

[1] Department of Obstetrics and Gynecology, IVF Unit, Ruijin Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China

Corresponding Author:
Aijun Zhang, Department of Obstetrics and Gynecology, IVF Unit, Ruijin Hospital Affiliated to Shanghai Jiaotong University, 197, Ruijin No. 2 Road, Shanghai, China.
Email: ivfruijin@yeah.net

10 kDa protein, IL-1β, and tumor necrosis factor-α (TNF-α) levels in endometrial secretions and implantation or clinical pregnancy in IVF–embryo transfer cycles.[12,13]

As a part of a research series on endometrium receptivity in adenomyosis, we used multiplex immunoassays, immunohistochemistry staining, and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) to clarify the inflammatory cytokine profile of endometrium in patients with adenomyosis during the implantation window after ovarian stimulation.

Materials and Methods

This study included 42 women who were undergoing their first cycle of IVF/ICSI during May 2012 to March 2013 in the Reproductive Medical Center of Ruijin Hospital affiliated with Shanghai Jiaotong University, Shanghai, People's Republic of China. The exclusion criteria for the study were the presence of hydrosalpinges, uterine fibroids, endometriosis, and previous myomectomy. All patients included had not habits of alcoholism, smoking, or drugs, without receiving hormone or anti-inflammatory medications at least 1 year before the study.

Before commencing their treatment, all patients underwent Transverginal ultrasound (TVS) in the follicular phase of the menstrual cycle, performed by 2 investigators experienced in gynecologic imaging. The ultrasound machine used was an Aloka Prosound SSD 3500 (Aloka Co Ltd, Tokyo, Japan). The diagnosis of focal or diffuse adenomyosis was established by applying 3 or more of the following established sonographic criteria[14]:

- asymmetry of uterine walls without leiomyomas;
- myometrial areas of heterogeneous echogenicity with poorly defined borders;
- minimal mass effect on the endometrium or serosa relative to the size of the myometrial lesion;
- small myometrial cysts or hemorrhagic foci within the heterotopic endometrial tissue;
- echogenic nodules or linear striations radiating from the endometrium into the myometrium; and
- absence of circular vascularization (determined by color Doppler) at the border of the lesion as usually observed with fibroids.

Among these patients, 18 were diagnosed with adenomyosis and the other 24 patients without evidence of adenomyosis were used as controls in this study. Written informed consent was obtained from all subjects before participation, and the Human Ethics Committee of Ruijin Hospital approved the protocol.

All patients received the regular gonadotropin-releasing hormone (GnRH) antagonist protocol for ovarian stimulation to obtain multiple oocytes. Briefly, daily injections of recombinant follicle-stimulating hormone (FSH; 100-300 IU/d) were given from cycle day 2 or 3 of a spontaneous cycle. On stimulation day 6, a GnRH antagonist (ganirelix acetate; Ganirelix Acetate Injection/Orgalutran; Merck/MSD [Cetrorelix Acetate, Merck, France]) was initiated at a daily dose of 0.125 mg and continued throughout the stimulation period. Final oocyte maturation was triggered by a single subcutaneous injection of human chorionic gonadotropin (hCG) of 5000 IU. Oocyte retrieval was carried out transvaginally under ultrasound guidance 34 to 36 hours after hCG injection. None of the patients in this study underwent embryo transfer after IVF/ICSI treatment: patients with adenomyosis for probable decreased endometrium receptivity[15] and the control group for fertilization failure.

Endometrial Sampling and Processing

Endometrial samples were obtained 7 days after hCG injection (hCG + 7). First, uterine secretions were aspirated: with the patient lying in the lithotomy position, the cervix was cleansed after insertion of the speculum and an embryo transfer catheter (Wallace; SIMS Portex Ltd, Hythe, Kent, United Kingdom) was introduced transcervically. Next, suction was gradually applied with a 2-mL syringe. To prevent contamination from the cervical mucus during catheter removal, the outer sheath of the embryo transfer catheter was advanced to a depth of 4 cm from the external cervical opening (os) following suction application. The inner catheter was then withdrawn through the outer sheath, which prevented contact with the cervix. The outside of the catheter was cleaned to remove any potential cervical mucus. The tips of the catheter were cutoff and snap frozen in liquid nitrogen and stored at −80°C until further use.

Endometrial biopsy samples were obtained using endometrial Pipelle catheters (CCD Laboratories, Paris, France) immediately after uterine aspiration. Each biopsy yielded approximately 1 mL of tissue. Half of this tissue was frozen on dry ice and stored at −80°C until further processing, while the other half was fixed in 10% neutral-buffered formalin and subsequently paraffin embedded.

Multiplex Immunoassay

The endometrial secretions samples were analyzed using a multiplex immunoassay. Key soluble regulators of implantation were identified as candidate mediators for inclusion in the assay. The panel included IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IL-18, TNF-α, IFN-γ, granulocyte-colony stimulating factor (G-CSF), and MCP-1. Assays were performed as described previously.[16] Every endometrium secretion sample was assayed twice with the average value as finial result.

Immunohistochemistry

Sections cut from the paraffin-embedded tissues were stained with hematoxylin and eosin for histological dating by a gynecologic pathologist to confirm that the samples were consistent with the early receptive period. Immunolabeling was carried out using the avidin–biotin–peroxidase complex method as described previously[8] Endometrial epithelial and stromal cells were counted and scored separately using the H-score value

Table 1. Primer Sequences and TaqMan Probes Used in Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction.

	Primer/Probe	Sequence
IL-6	Forward	5′-TACCCCCAGGAGAAGATTCC-3′
	Reverse	5′-TTTTCTGCCAGTGCCTCTTT-3′
IL-10	Forward	5′-ATGCACAGCTCAGCACTGC-3′
	Reverse	5′-TCAGTTTCGTATCTTCATTGTC-3′
IL-17	Forward	5′-CAAGACTGAACACCGACTAAG-3′
	Reverse	5′-TCTCCAAAGGAAGCCTGA-3′
INF-r	Forward	5′-AGTTATATCTTGGCTTTTCA-3′
	Reverse	5′-ACCGAATAATTAGTCAGCTT-3′
MCP-1	Forward	5′-AACTGAAGCTCGCACTCTCG-3′
	Reverse	5′-TCAGCACAGATCTCCTTGGC-3′

Abbreviations: IL, interleukin; MCP-1, monocyte chemoattractant protein-1; INF-r, interferon-r.

method, described in detail elsewhere.[17] Immunohistochemistry slides were scored by 2 experienced pathologists of different hospitals while the data collector and sample processing staff were double blinded.

Quantitative Analysis of Messenger RNA

Total RNA was isolated from endometrial cells using the Trizol reagent (Sigma, St Louis, MO) following the manufacturer's protocol. RNA (1 mg) was used to generate complimentary DNA with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, California). The qRT-PCR was performed with the ABI 7900 Sequence Detection System and the ABI TaqMan Gene Expression system (Applied Biosystems, Foster City, California). The primer sequences are summarized in Table 1. Relative quantification of messenger RNA (mRNA) species' was performed using the comparative threshold cycles (Ct) method.

Data Analysis

The sample size was estimated using the method recommended by Cohen.[18] The present sample size (24 controls and 18 with adenomyosis) is enough to discard at least a large effect size (0.83 standard deviations) between these 2 groups with a power of 80% and α of 5%. We log-transformed nonnormally distributed data prior to analysis. Primary analyses were performed using the SPSS version 14.0 (SPSS Inc, Chicago, Illinois) considering a value of $P < .05$ statistically significant. Data are presented as mean $\pm$ standard error of the mean, and data from the 2 groups were compared using the Student t test or Mann-Whitney U test. Categorical variables were compared using the chi-square test and Fisher exact test.

Results

Clinical Data

A total of 42 patients undergoing IVF/ICSI stimulation cycle were included in this study, including 18 patients with adenomyosis and 24 patients with no evidence of adenomyosis. A total of 238 women were assessed for eligibility to this study, and 196 were excluded for reasons given in flow chat Figure 1. Table 2 compares the baseline demographic and clinical variables between the patients with or without adenomyosis; no differences between the 2 groups in terms of age, body mass index, or basal sex hormone levels (FSH, luteinizing hormone [LH], and estradiol [E2]) were found. The LH, E2, and progesterone levels on HCG administration day and the number of oocytes retrieved also had no significant difference. However, the group with adenomyosis had thicker endometrium on hCG trigger day and a greater proportion of dysmenorrhea compared to the group without adenomyosis. In addition, serum CA125 levels were significantly higher in the adenomyosis group.

Endometrium Secretion Multiplex Immunoassay

Of the 42 women who underwent endometrial secretion aspiration, 1 patient was excluded because insufficient material was available to measure total protein content in the aspirate. Twelve mediators (IL-1, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13 IL-17, IFN-γ, MCP-1, and G-CSF) were detectable in over 90% of the samples. The IL-4, IL-5, IL-18, and TNF-α were detectable in 23.8%, 21.4%, 35.7%, and 59.5% of patients, respectively. Significantly different concentrations of IL-6, IL-10, IL-17, IFN-γ, and MCP-1 ($P < .05$) in endometrial secretions in women with adenomyosis versus the control group were observed (Figure 2). The IL-6, IFN-γ, and MCP-1 levels in endometrial secretions from patients with adenomyosis were higher, while IL-10 and IL-17 levels were lower compared to the control group.

Immunohistochemistry and qRT-PCR

Based on the results from the multiplex immunoassay, we choose IL-6, IL-10, IL-17, IFN-γ, MCP-1, and CD-68+ (indicating the presence of macrophages) for the endometrium immunohistochemistry staining assay. Both in epithelium and stroma, CD-68, IL-6, and MCP-1 immunolabeling H-scores were significantly higher in adenomyosis endometrium epithelia than controls. Conversely, IL-10 immunolabeling H-scores in endometria were significantly lower in the adenomyosis group compared to the control group (Figure 3 and Table 3). The qRT-PCR corroborated the immunohistochemistry results. There were no significant differences in IL-17 or IFN-γ expression, either by H-score or qRT-PCR.

Relationship Between Multiplex Immunoassay and Immunohistochemistry

In patients with adenomyosis, we also found that IL-6, IL-10, and MCP-1 levels in endometrium secretions determined by multiplex immunoassay were significantly correlated with expression in endometrium epithelium indicated by H-score using Spearman correlation assay. There was also a significantly positive coefficient between MCP-1 and CD-68+

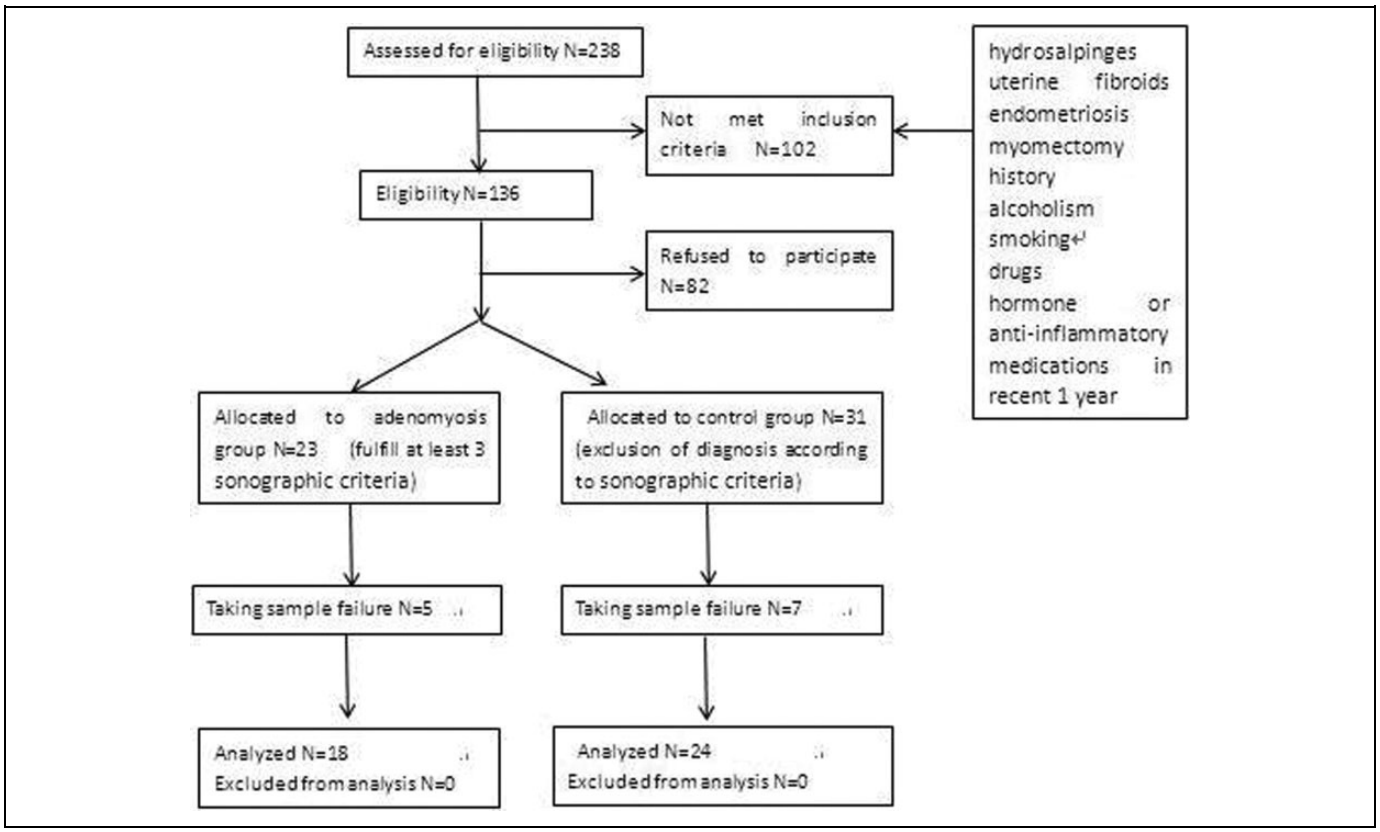

Figure 1. Participant flow chart for the study of endometrium of adenomyosis versus control after ovarian simulation.

Table 2. Subject Baseline Characteristics.

	Adenomyosis	Control	*P* Value
Number subjects	18	24	
Age, years	30.9 ± 4.3	31.4 ± 3.9	.14
Body mass index, kg/m^2	21.6 ± 2.9	21.7 ± 3.2	.25
Basal follicle-stimulating hormone level, IU/mL	7.8 ± 2.0	8.1 ± 1.4	.17
Basal luteinizing hormone level, IU/mL	6.0 ± 4.1	6.2 ± 3.4	.19
Basal estradiol level, pg/mL	26.3 ± 11.9	34.2 ± 13.9	.22
Endometrium thickness, mm	12.6 ± 1.9	10.2 ± 1.5	.04
Serum cancer antigen 125, IU/mL	64.0 ± 28.0	12.3 ± 8.2	.02
Dysmenorrhea, %	100	12.5	.01
Estrogen level on hCG day, pg/mL	3950 ± 1326.8	4238 ± 1409.6	.11
Luteinizing hormone level on hCG day, pg/mL	2.8 ± 1.2	3.1 ± 1.5	.09
Progesterone level on hCG day, ng/mL	1.1 ± 0.3	1.0 ± 0.4	.10
No. of oocyte retrieved	9.6 ± 2.7	10.5 ± 3.1	.12

Abbreviations: hCG, human chorionic gonadotropin; INF-r, interferon-r.

H-score in the endometrium epithelium of patients with adenomyosis (Figure 4).

Discussion

Over the past 3 decades, tremendous progresses has been made in the diagnosis of adenomyosis using imaging techniques and, at present, magnetic resonance imaging and transvaginal sonography provide comparably good diagnostic results. The application of noninvasive diagnostic technology has revealed that adenomyosis is present even in younger women and is associated with pelvic endometriosis and infertility.[19] While the exact mechanisms by which adenomyosis limits fertility are still under debate, alterations in the endometrial immune environment was suggested as a possible cause in a small case series of patients with adenomyosis.[20]

During the preimplantation period, uterine glands produce a glycoprotein-rich secretion, which is believed to support the

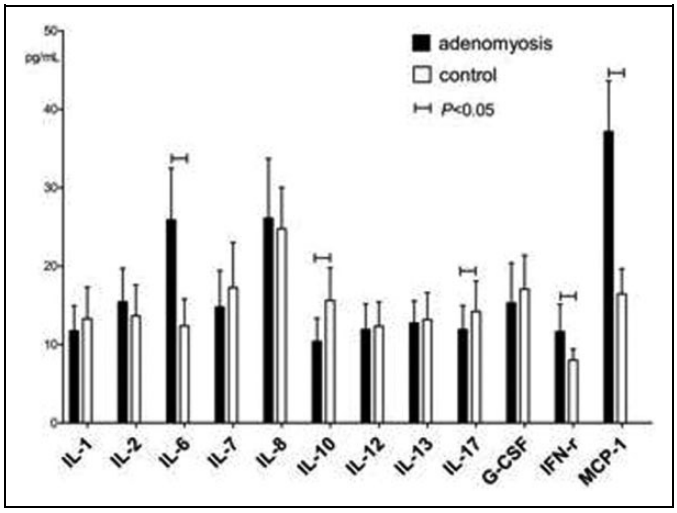

Figure 2. Comparison of cytokine levels in endometrium secretion from women with and without adenomyosis by multiplex immunoassay. Levels of interleukin-6 (25.9 ± 6.6 vs 12.4 ± 3.4 pg/mL; $P = .001$), interleukin-10 (10.4 ± 2.9 vs 15.6 ± 4.2 pg/mL; $P = .001$), interleukin-17 (11.9 ± 3.0 vs 14.2 ± 3.9 pg/mL; $P = .046$), interleukin-γ (11.7 ± 3.5 vs 8.0 ± 3.4 pg/mL; $P = .001$), and MCP-1 (37.1 ± 6.5 vs 16.4 ± 3.2 pg/mL; $P = .001$) were significantly different between patients with adenomyosis and control groups, respectively.

embryo.[21] In addition to providing nutrition for the conceptus, it contains a complex array of growth factors and cytokines that act as autocrine and paracrine messengers between endometrial cells and leukocytes. Modern multiplex immunoassays enable multiple markers to be quantified simultaneously using a small sample volume.[16] Some studies have shown this approach not only to be nondisruptive to implantation but also representative of the in vivo milieu encountered by the embryo.[9,10]

In the present study, we assayed a set of cytokines from the uterine microsurroundings during the implantation window of the ovarian stimulation cycle of adenomyosis and control patients by multiplex immunoassay. We show here, for the first time, significantly higher IL-6, IFN-γ, and MCP-1 levels and lower concentrations of IL-10 and IL-17 in endometrial secretions from patients with adenomyosis compared to normal controls. Further, we used immunohistochemistry and qRT-PCR to verify the results of the multiplex immunoassays. Through this process, we identified differential expression of macrophages, IL-6, IL-10, and MCP-1 in endometrium between patients with adenomyosis and control patients.

Based on previous endometriosis research, it was concluded that endometrial implantation outside of utero appear to activate host immune responses, leading to hormonal- and cell-mediated inflammation.[22] As with endometriosis, some published findings suggest that an abnormal inflammatory response may be

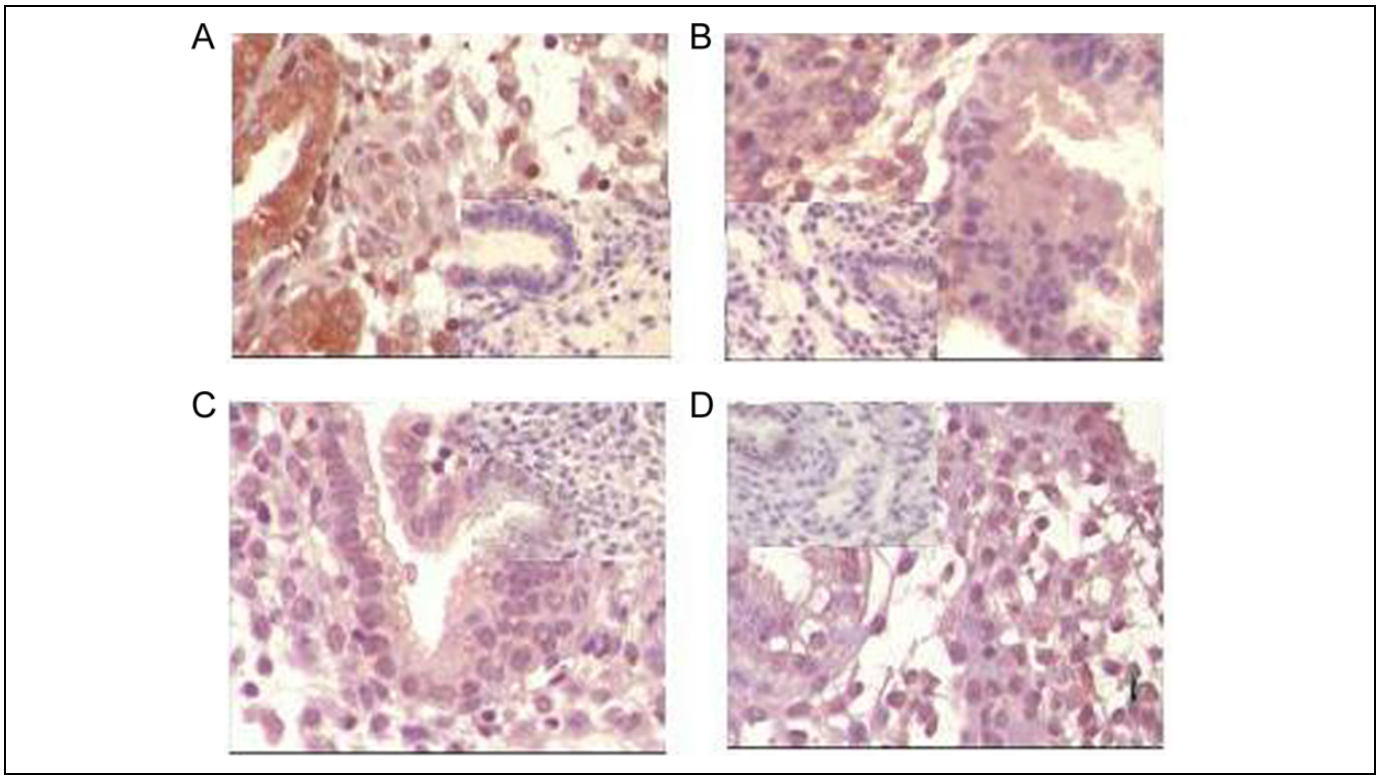

Figure 3. Immunohistochemistry study of CD-68, IL-6, IL-10, and MCP-1 expressions at the level of EMI in uterine adenomyosis. Immunoreactive CD-68 (A), IL-6 (B), IL-10 (C), and MCP-1 (D) were identified in the human eutopic endometrium at the level of EMI using HRP staining. A brown reaction product indicated a positive reaction for antibody staining. The inset photographs are negative control of omitting the primary antibodies for matched tissues and targets (×400). IL indicates interleukin; MCP-1, monocyte chemoattractant protein-1; EMI, endometrial myometrial interface; HRP, horseradish peroxidase.

Table 3. The Comparison of Expression of CD-68, IL-6, IL-10, IL-17, IFN-γ, and MCP-1 in Endometrium of Adenomyosis and the Control Group (Mean ± Standard Error of the Mean).

	Adenomyosis			Control		
	H-score			H-score		
	Epithelium	Stroma	mRNA	Epithelium	Stroma	mRNA
CD-68[a]	12.56 ± 2.75	5.44 ± 2.40	NA	7.56 ± 3.07	1.65 ± 0.63	NA
IL-6[b]	10.33 ± 2.71	8.33 ± 3.24	0.83 ± 0.23	6.30 ± 1.95	2.6 ± 0.74	0.62 ± 0.13
IL-10[b]	6.83 ± 1.38	1.67 ± 0.43	0.76 ± 0.19	10.3 ± 1.7	7.25 ± 1.44	0.53 ± 0.12
IL-17	9.26 ± 2.45	3.54 ± 1.96	0.64 ± 0.35	10.18 ± 2.96	3.15 ± 2.18	0.59 ± 0.15
INF-r	8.37 ± 2.48	2.36 ± 1.87	0.77 ± 0.27	7.26 ± 1.98	2.85 ± 1.99	0.68 ± 0.23
MCP-1[b]	13.39 ± 2.92	3.89 ± 1.12	1.38 ± 0.29	6.12 ± 1.96	2.35 ± 1.38	1.15 ± 0.22

Abbreviations: IL, interleukin; IFN, interferon; MCP-1, monocyte chemoattractant protein-1; mRNA, messenger RNA.
[a]H-score (epithelium and stroma) of adenomyosis versus control, $P < .05$, no mRNA data.
[b]H-score (epithelium and stroma) and mRNA expression of adenomyosis versus control, $P < .05$.

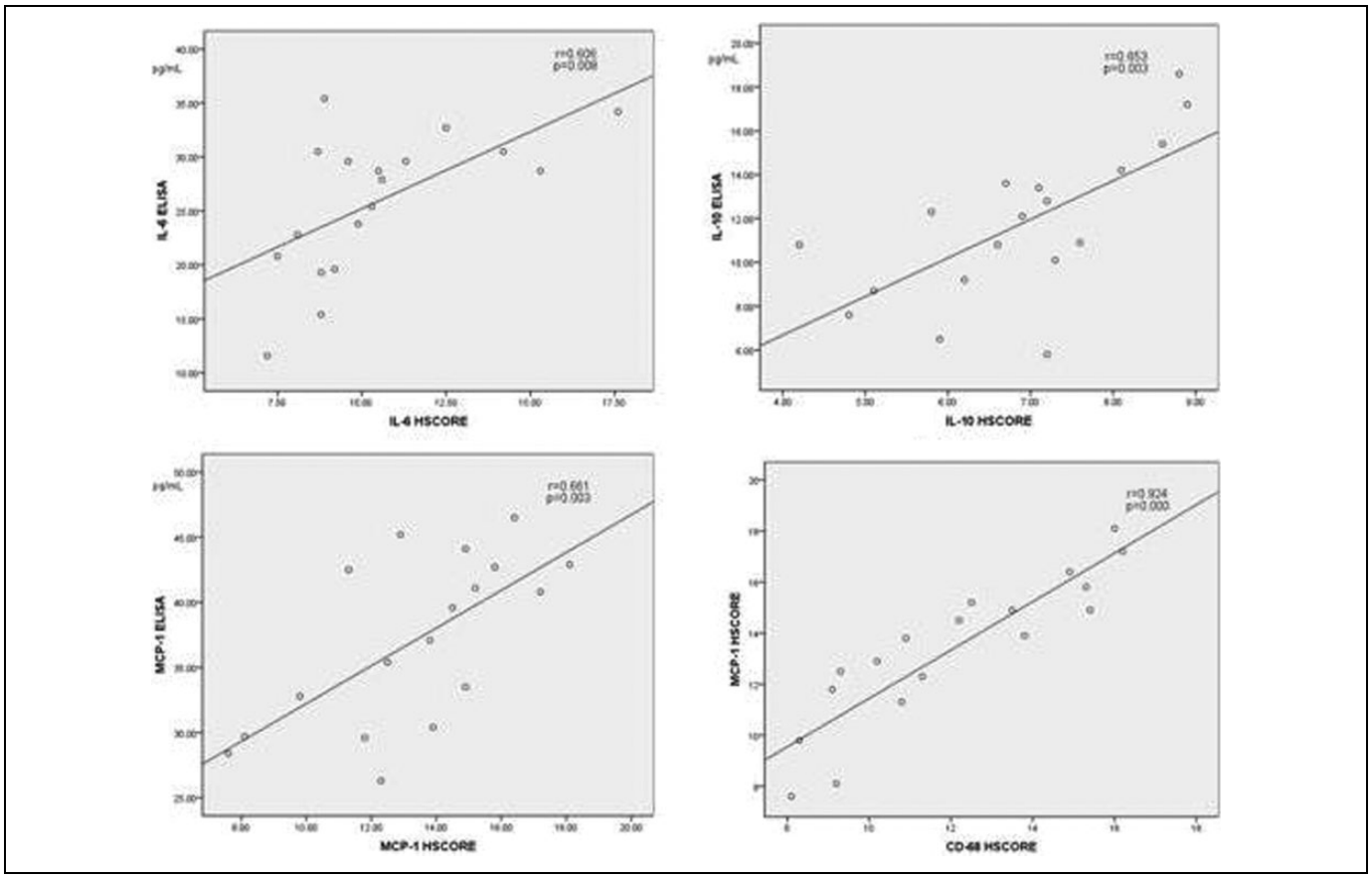

Figure 4. Relationships between endometrium secretion levels of interleukin-6, interleukin-10, and monocyte chemotactic protein-1 assayed by multiplex immunoassay and H-score of interleukin-6, interleukin-10, monocyte chemotactic protein-1, and cluster of differentiation-68 in endometrium epithelium by immunohistochemistry.

present in eutopic and ectopic endometrium of women with adenomyosis and this may impair fertility because the population of immunocompetent cells at the endometrial–myometrial interface is disrupted.[23-25] In the present study, we found a significantly increased number of macrophages in adenomyosis endometrium epithelia compared to controls as determined by immunohistochemistry. In line with our results, some other published studies reported excessively high endometrial macrophage density in adenomyosis women compared to normal controls.[26,27] It is well recognized that macrophages have the capability of releasing embryotoxic cytokines, reactive oxygen species, and nitrogen species,[28-30] which can directly damage embryos and have been reported to increase local production of prostaglandin F2α, leading to an increase in myometrial

contractility that is likely to further impede the implantation process. A recent study has reported that long-term GnRH agonist downregulation therapy can produce a very significant fall in endometrial macrophage numbers with a coexistent decline in endometrial tissue production of MCP-1, a cytokine known to be chemotactic for macrophages.[31] Based on these data, it was postulated that excess estrogen action in the endometrium of adenomyosis may result in an increase production of proinflammatory cytokines, resulting in an elevated endometrial macrophage density.

Large populations of immune cells accumulate in the endometrium during the luteal phase and are believed to be important modulators of implantation. Among these, IL-6 carries out important functions in reproductive physiology, including the regulation of ovarian steroid production, folliculogenesis, and early events related to implantation.[32,33] In the human endometrium, IL-6 is weakly produced by luminal and glandular epithelial cells in the proliferative phase; maximal expression occurs in both the epithelium and the stroma in the mid-secretory phase at the time of implantation, while IL-6 receptor subunit remains unchanged. It has been postulated that IL-6 might play a role in the formation of ectopic endometrial implants in adenomyosis because IL-6 mRNA expression was significantly increased in endometrial stromal cells (ESCs) after in vitro coculture with macrophages in adenomyosis.[34] This abnormal production of IL-6 in eutopic endometrium was also found in women with endometriosis, in which the IL-6 secretion was higher in eutopic ESCs than in healthy controls.[35]

The critical pathophysiologic mechanism of IL-6 in the development of adenomyosis might be angiogenesis because IL-6 is a potent angiogenic factor; angiogenesis triggered by IL-6 has been found in human cervical cancer cells to act through the signal transducer and activator of transcription 3 pathway, which might subsequently promote cervical tumorigenesis.[36]

The IL-10, an important anti-inflammatory and immunosuppressive cytokine, is constitutively locally produced by trophoblasts, uterine natural killer (NK) cells, and macrophages[37-39] in the context of pregnancy. In placental tissue obtained from normal pregnant women, immunohistochemical analysis coupled with enzyme-linked immunosorbent assay showed that IL-10 was produced in a gestational age-dependent manner.[40] No related literature is available with regard to the mechanism of IL-10 interference with local and total immune response in patients with adenomyosis. However, it has been proven that IL-10, as a potent antiangiogenic factor, inhibits inflammation-mediated vascular dysfunction that orchestrates proper pregnancy outcomes.[41] Several studies have associated IL-10 deficiency to recurrent spontaneous abortion.[42,43] According to research by Bates et al,[44] spontaneous abortions have been correlated with decreased production of IL-10 by peripheral blood mononuclear cells and a significant decrease in IL-10 expression in the endometrium was also seen during the peri-implantation period in women that experienced recurrent spontaneous abortions compared to controls.[43,45] Administration of recombinant IL-10 in abortion prone CBA $\times$ DBA/2 mice significantly abrogated the incidence of spontaneous fetal loss.[46] Thus, decreased expression of IL-10 in adenomyosis uteri, as our shown in our results, may be associated with impaired endometrium receptivity in these patients.

The MCP-1 is a β-chemokine that specifically induces chemotaxis and activation of mononuclear phagocytes. This chemokine is produced by a number of cells, including endothelial cells, fibroblasts, monocytes, lymphocytes, smooth muscle cells, and even tumor cells.[47,48] Studies have revealed that MCP-1 may influence both innate immunity, through its effects on monocytes, and adaptive immunity, through its control of T helper cell polarization.[49] In human reproduction, MCP-1 may play a pivotal role in immune recognition, acceptance of the fetal allograft, maintenance of pregnancy, and parturition.[50] According to published in vitro and in vivo studies, the endometrial cells of women with endometriosis abnormally express increased levels of MCP-1 both in local (peritoneal fluid and endometriotic lesions) and systemic (peripheral blood) contexts.[51,52] It was speculated that, in the pathogenesis of endomyosis, MCP-1 may play a fundamental role in the early development and maintenance of ectopic endometrium by stimulating the attachment of endometrial cells to the extracellular matrix and induce proliferation of endometriotic cells.[53,54] Since adenomyosis is closely related to endometriosis and our results also showed increased levels of MCP-1 in the uteri cavities and endometrium of patients with adenomyosis compared to controls during the secretory phase, we postulate that MCP-1 may be a major immune factor involved in the pathogenesis of adenomyosis.

Moreover, MCP-1 has been shown to be a potent attractant and activator of uterine (u) NK cells.[55] High numbers of uNK cells have been related to miscarriage and infertility,[56] which is consistent with higher secretion levels of MCP-1 being associated with lower implantation rates, such as in patients with adenomyosis. Boomsma et al applied multiplex immunoassay technology to identify cytokine profiles of endometrium secretion for predicting pregnancy in IVF.[13] Their results indicate that initial embryo implantation in IVF was significantly negatively associated with the concentration of endometrial MCP-1 secretion.

In the ovarian stimulation cycle, supraphysiological levels of estrogen caused the increased presence of uNK cells, which can secrete an array of cytokines in the luteal phase,[55-57] including IL-17 and IFN-γ. In the present study, lower levels of IL-17 and higher levels IFN-γ in the uteri of patients with adenomyosis determined by multiplex immunoassay were not confirmed by immunohistochemistry and qRT-PCR. We hypothesize that the reason for these different results may be attributed to the different forms of the cytokines (secreted soluble or membrane-bound form) measured by the different assay technologies (immunoassay and immunohistochemistry, respectively). On the other hand, undetectable blood contamination in the samples may have affected the readings of some cytokines due to hemoglobin interfering with the laser readings. Therefore, blood contamination, a major limitation of this technology, should be included as a confounder when analyzing the results from cytokine measurements.

However, there are also a number of limitations to this study. The observational nature of this study is disadvantaged methodologically by an inability to control completely for selection and confounding biases.[58] Despite being the first published study reporting on inflammatory cytokine profile of endometrium in adenomyosis, the number of patients with adenomyosis was small in comparison to controls. Therefore, analyzing some recorded ultrasound data to examine whether the severity of adenomyosis correlated with cytokine profile would have been interesting but meaningless due to the small number of patients with adenomyosis in the study. On the other hand, pregnancy outcomes were unavailable as all embryo transfer was canceled in ovarian stimulation cycles.

In conclusion, to our knowledge, this is the first study to investigate the expression of these cytokines in adenomyotic uteri after ovarian stimulation. Our results suggest that within the implantation window of ovarian stimulation cycles, IL-6, IFN-γ, MCP-1, IL-10, and IL-17 levels in endometrial secretions from patients with adenomyosis were significantly different from that of the control group. Macrophages, IL-6, IL-10, and MCP-1 mRNA or protein are expressed differently in the eutopic endometrium of women with adenomyosis compared to normal endometrium, and this may correlate with compromised endometrium receptivity in these patients. On the other hand, our results may provide clues to the pathogenesis of adenomyosis associated with immunotolerant and/or anti-inflammatory processes. However, further investigation is needed to assess the precise mechanism by which cytokines participate in the development of adenomyosis and the embryo implantation process.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Natural Science Foundation of Shanghai, China, No. 11411950104.

References

1. Ferenczy A. Pathophysiology of adenomyosis. *Hum Reprod Update*. 1998;4(2):312-322.
2. Matalliotakis IM, Katsikis IK, Panidis DK. Adenomyosis: what is the impact on fertility? *Curr Opin Obstet Gynecol*. 2005;17(1): 261-264.
3. Piver P, Maubon A, Kapella M, Gana J, Paulhiac S, Aubard Y. Magnetic resonance imaging evaluation of adenomyosis with in IVF patients' pregnancy rate is linked to measures of junction zone. *Fertil Steril*. 2003;80(1):S73-S74.
4. Shimizu Y, Fukuda J, Kawamura K, Tanaka T. Retrospectively analysis of the fertility of adenomyosis and the outcome of adenomyosis complicated pregnancy in in vitro fertilization patients. *Fertil Steril*. 2002;77(2):S50.
5. Wold AD, Patrizio P, Sepulveda J, Eduse A, Arici A. The impact of MRI confirmed diagnosis of adenomyosis on the success rates of infertility treatment. *Fertil Steril*. 2005;84(2):S192-S193.
6. Salim R, Riris S, Saab W, Abramov B, Khadum I, Serhal P. Adenomyosis reduces pregnancy rates in infertile women undergoing IVF. *RBM Online*. 2012;25(5):l273-1277.
7. Ota H, Tanaka T. Stromal vascularization in the endometrium during adenomyosis. *Microsc Res Tech*. 2003;60(2):445-449.
8. Ota H, Igarashi S, Hatazawa J, Tanaka T. Immunohistochemical assessment of superoxide dismutase expression in endometriosis and adenomyosis. *Fertil Steril*. 1999;72(1):129-134.
9. Van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, Macklon NS. Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates. *RBM Online*. 2003; 7(1):105-109.
10. Boomsma CM, Kavelaars A, Eijkemans MJ, et al. Cytokine profiling in endometrial secretions: a noninvasive window on endometrial receptivity. *RBM Online*. 2009;18(1):85-94.
11. Campo S, Campo V, Benagiano G. Adenomyosis and infertility. *Reprod Biomed online*. 2012;24(1):35-46.
12. Boomsma CM, Kavelaars A, Eijkemans MJC, et al. Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in in-vitro fertilization. *Hum Reprod*. 2009; 24(1):1-9.
13. Boomsma CM, Kavelaars A, Eijkemans MJ, Fauser BC, Heijnen CJ, Macklon NS. Ovarian stimulation for in vitro fertilization alters the intrauterine cytokine, chemokine, and growth factor milieu encountered by the embryo. *Fertil Steril*. 2010;94(6): 1764-1768.
14. Dueholm M, Lundorf E. Transvaginal ultrasound or MRI for diagnosis of adenomyosis. *Curr Opin Obstet Gynecol*. 2007;19(2): 505-512.
15. Niu ZH, Chen Q, Sun YJ, Feng Y. Long-term pituitary downregulation before frozen embryo transfer could improve pregnancy outcomes in women with adenomyosis. *Gynecol Endocrinol*. 2013;29(4):1026-1030.
16. de Jager W, te Velthuis VH, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol*. 2003;10(1):133-139.
17. McCarty KS Jr, Szabo E, Flowers JL, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res*. 1986;46(8):4244-4248.
18. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*. 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.
19. Camargo F, Gaytan J, Caligara C, Simon C, Pellicer A, Remohi J. Impact of ultrasound diagnosis of adenomyosis on recipients of sibling oocytes. *Fertil Steril*. 2001;76(2):S150.
20. Ota H, Igarashi S, Hatazawa J, Tanaka T. Is adenomyosis an immune disease? *Hum Reprod Update*. 1998;4(2):360-367.
21. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *J Clin Endocrinol Metab*. 2002;87(7):2954-2959.
22. Wu MY, Ho HN. The role of cytokines in endometriosis. *Am J Reprod Immunol*. 2003;49(1):285-296.

23. Wang F, Li H, Yang Z, Du X, Cui M, Wen Z. Expression of interleukin-10 in patients with adenomyosis. *Fertil Steril*. 2009; 91(5):1681-1685.
24. Yang JH, Wu MY, Chang DY, Chang CH, Yang YS, Ho HN. Increased interleukin-6 messenger RNA expression in macrophage co-cultured endometrial stromal cells in adenomyosis. *Am J Reprod Immunol*. 2006;55(1):181-187.
25. Yen CF, Basar M, Kizilay G, Lee CL, Kayisli UA, Arici A. Implantation markers are decreased in endometrium of women with adenomyosis during the implantation windows. *Fertil Steril*. 2006;86(2):550-557.
26. Tremellen K, Russell P. Adenomyosis is a potential cause of recurrent implantation failure during IVF treatment. *Aust N Z J Obstet Gynaecol*. 2011;51(1):280-283.
27. Tremellen KP, Russell P. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. II: adenomyosis and macrophages. *J Reprod Immunol*. 2012;93(1):58-63.
28. Agarwal A., Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol*. 2005;3(1):28-35.
29. Lee TH, Wu MY, Chen MJ, Chao KH, Ho HN, Yang YS. Nitric oxide is associated with poor embryo quality and pregnancy outcome in in vitro fertilization cycles. *Fertil Steril*. 2004;82(1): 126-131.
30. Haddad EK, Duclos AJ, Baines MG. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. *J Exp Med*. 1995;182(4):1143-1151.
31. Khan KN, Kitajima M, Hiraki K, et al. Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy. *Hum Reprod*. 2010;25(2):642-653.
32. Gorospe WC, Hughes FM Jr, Spangelo BL. Interleukin-6: effects on and production by rat granulosa cells in vitro. *Endocrinology*. 1992;130(4):1750-1752.
33. Jacobs AL, Sehgal PB, Julian J, Carson DD. Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro. *Endocrinology*. 1992;131(2):1037-1046.
34. Yang JH, Wu MY, Chang DY, Chang CH, Yang YS, Ho HN. Increased interleukin-6 messenger RNA expression in macrophage-cocultured endometrial stromal cells in adenomyosis. *Am J Reprod Immunol*. 2006;55(1):181-187.
35. Tseng JF, Ryan IP, Milam TD, et al. Interleukin-6 secretion in vitro is up-regulated in ectopic and eutopic endometrial stromal cells from women with endometriosis. *J Clin Endocrinol Metab*. 1996;81(3):1118-1122.
36. Wei LH, Kuo ML, Chen CA, et al. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene*. 2003;22:(3)1517-1527.
37. Lidstrom CL, Matthiesen G, Berg S, Sharma J, Ernerudh C. Cytokine secretion patterns of NK cells and macrophages in early human pregnancy decidua and blood: implications for suppressor macrophages in decidua. *Am J Reprod Immunol*. 2003;50(1): 444-452.
38. Thaxton JE, Sharma S. Interleukin-10: a multi-faceted agent of pregnancy. *Am J Reprod Immunol*. 2010;63(1):482-491.
39. Roth I, Corry DB, Locksley RM, Abrams JS, Litton MJ, Fisher SJ. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. *J Exp Med*. 1996;184(2):539-548.
40. Hanna N, Hanna I, Hleb M, et al. Gestational age dependent expression of IL-10 and its receptor in human placental tissues and isolated cytotrophoblasts. *J Immunol*. 2000;164(7): 5721-5728.
41. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 2001;19(1):683-765.
42. Raghupathy R, Makhseed M, Azizieh F, et al. Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. *Cell Immunol*. 1999;196(1):122-130.
43. Plevyak M, Hanna N, Mayer S, et al. Deficiency of decidual IL-10 in first trimester missed abortion: a lack of correlation with the decidual immune cell profile. *Am J Reprod Immunol*. 2002; 47(1):242-250.
44. Bates MD, Quenby S, Takakuwa K, Johnson PM, Vince GS. Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? *Hum Reprod*. 2002;17(9): 2439-2444.
45. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *J Clin Endocrinol Metab*. 2002;87(8):2954-2959.
46. Chaouat G, Assal Meliani A, Martal J, et al. IL-10 prevents naturally occurring fetal loss in the CBAxDBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is correction by in vivo injection of IFN-tau. *J Immunol*. 1995;154(7):4261-4268.
47. Sozen I, Olive DL, Arici A. Expression and hormonal regulation of monocyte chemotactic protein-1 in myometrium and leiomyomata. *Fertil Steril*. 1998;69(3):1095-1102.
48. Hampton AL, Rogers PA, Affandi B, Salamonsen LA. Expression of the chemokines, monocyte chemotactic protein (MCP)-1 and MCP-2 in endometrium of normal women and Norplant users, does not support a central role in macrophage infiltration into endometrium. *J Reprod Immunol*. 2001;49(1):115-132.
49. Akoum A, Turcot-Lemay L, Lemay A, Maheux R, McColl S. Elevated concentration and biologic activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. *Fertil Steril*. 1996;66(1):17-23.
50. Ones RL, Hannan NJ, Kaitu'u TJ, Zhang J, Salamonsen LA. Identification of chemokines important for leukocyte recruitment to the human endometrium at the times of embryo implantation and menstruation. *J Clin Endocrinol Metab*. 2004;89(10):6155-6167.
51. Akoum A, Lemay A, Brunet C, Hébert J. Secretion of monocyte chemotactic protein-1 by cytokine-stimulated endometrial cells of women with endometriosis. *Fertil Steril*. 1995;63(1):322-328.
52. Jolicoeur C, Boutouil M, Drouin R, Paradis I, Lemay A, Akoum A. Increased expression of monocyte chemotactic protein-1 in the endometrium of women with endometriosis. *Am J Pathol*. 1998; 152(1):125-133.
53. Ulukus M, Ulukus C, Tavmergen EN, Tavmergen E, Zheng WX, Arici A. Expression of interleukin-8 and monocyte chemotactic

protein 1 in women with endometriosis. *Fertil Steril*. 2009;91(3): 687-693.
54. Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. Monocyte chemotactic proteins MCP-1, MCP-2 and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. *FASEB J*. 1994;8(3):1055-1060.
55. DeLoia JA, Stewart-Akers AM, Brekosky J, Kubik CJ. Effects of exogenous estrogen on uterine leukocyte recruitment. *Fertil Steril*. 2002;77(2):548-554.
56. Lukassen HG, Joosten I, van Cranenbroek B, et al. Hormonal stimulation for IVF treatment positively affects the CD56bright/CD56dim NK cell ratio of the endometrium during the window of implantation. *Mol Hum Reprod*. 2004;10(2):513-520.
57. Quenby S, Farquharson R. Uterine natural killer cells, implantation failure and recurrent miscarriage. *RBM Online*. 2006;13(1): 24-28.
58. Greenhalgh T. Assessing the methodological quality of published papers. *Br Med J*. 1997;315(1):305-308.