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ORIGINAL ARTICLE

Serum and Peritoneal Fluid Cytokine Profiles in Infertile Women with Endometriosis

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ABSTRACT

Background: Endometriosis is a chronic inflammatory disease with the growth of endometrial cells out of uterus and in the peritoneal cavity. T cell subsets participate in the establishment and progress of the disease by producing different cytokines. **Objective:** To investigate a group of cytokines related to Th1/Th2/Th17/Treg subsets within both peripheral blood and peritoneal fluid (PF) samples from infertile endometriosis women. **Methods:** Peripheral blood and PF samples were collected from 30 infertile endometriosis and 30 non-endometriosis fertile women during laparoscopy. Concentration of cytokines, including TNF-α, IFN-γ, TGF-β1, IL-4, IL-10, IL-17 and IL-23 were evaluated using ELISA method. **Results:** Results indicated that the concentration of IFN-γ within serum was significantly reduced in endometriosis group ($p=0.001$). Regarding PF cytokines, TGF-β1 was increased in endometriosis group ($p=0.030$). Furthermore, the ratios of IFN-γ/TGF-β1 and IL-17/IL-23 were significantly different between endometriosis and non-endometriosis women in serum samples ($p<0.001$ and $p<0.01$ respectively). The ratios of TNF-α/IL-10 and IL-17/IL-10 were also significantly different regarding PF samples between the two studied groups ($p<0.04$ and $p<0.03$ respectively). Finally, significant correlations were observed between the levels of IL-17 and IL-23, inflammatory and anti-inflammatory cytokines, in both samples and serum to PF inflammatory cytokines. **Conclusion:** Based on the results of the present study, in women with endometriosis, the disturbance of cytokines network might gradually activate the inflammatory responses and tissue repair, resulting in endometriosis development after several years.

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INTRODUCTION

Endometriosis is a chronic inflammatory disease defined as the presence and growth of endometrial glands and stroma outside the uterine cavity (1). Endometriosis is considered as a common gynecological disorder which manifests with pelvic pain, dysmenorrhea, and infertility (2). This disease is one of the main causes of hospitalization due to gynecological reasons. It is reported that race, age, body mass index, and smoking are associated with the incidence of endometriosis (3). The incidence rate for endometriosis is highest among women aged under 30 years and lowest among those over 40 years old (3). Even after adjusting for age, there is a significant reduction in the incidence of endometriosis with the increase in the body mass index (4). Although there is controversy regarding the exact etiology of the disease, it is believed that steroid hormones, and anatomic, genetic, and immunological factors play a major role in predisposition to endometriosis (3,4). There is strong evidence that the disturbances in immune responses may play important roles in the pathogenesis of endometriosis (5,6). Various immune cells are involved in the development of endometriosis, yet it is believed that T lymphocytes and their related cytokines are very important in disease development (7). Following stimulation and activation, T lymphocytes contribute to the conduction, progression, and regulation of the immune responses. Several published papers have reported the elevated levels of pro-inflammatory cytokines related to T helper 1 (Th1) subset within the peritoneal fluid (PF) in endometriosis women (8,9). The increased levels of Th1 cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IFN-γ in women with endometriosis have further been reported in the previous studies (10,11). The toxic effects of these inflammatory cytokines on female reproductive system, sperm, or embryo might cause infertility or subfertility observed in women with endometriosis (12,13). Several studies, on the other hand, have highlighted the role of Th2 responses and reported the elevated levels of IL-4 and IL-10 in women with endometriosis (1,14,15). In these reports, the imbalance between Th1 and Th2 responses was proposed as the basis in the pathogenesis of endometriosis (16). After the discovery of Th17 subset and its related inflammatory cytokines, including IL-17 and IL-23, Th17 subset was added to the previous puzzle (17). In this regard, Zhang and coworkers reported increased levels of IL-17 in women with early endometriosis (18). Besides, regulatory T cell (Treg) modulates the severity of the inflammatory responses via the production of regulatory cytokines such as IL-10 and TGF-β1 (19). Increased activities of Treg cells have been reported within the peritoneal fluid of women with endometriosis (20,21). There are also reports indicating the systemic changes in the levels of cytokines in the peripheral blood samples from endometriosis women (22,23). Previous reports regarding the levels of cytokines in endometriosis had limitations and controversial results. These reports mostly investigated a limited number of cytokines and did not compare their level in the peripheral blood with those from peritoneal fluid. Accordingly, the present study aimed to investigate a group of cytokines related to Th1/Th2/Th17/Treg subsets, including TNF-α, IFN-γ, IL-4, IL-10, IL-17, IL-23, and TGF-β1 within both PF and peripheral blood samples from infertile women with endometriosis and compare them with the concentration of the same cytokines in fertile non-endometriosis women. Moreover correlations between T helper subsets' cytokines were also investigated in the present study.

MATERIALS AND METHODS

Subjects. The present study was approved by the medical ethics committees of both Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.SM.REC.1394.79) and Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1395.S307). All participants read and signed a written informed consent prior to entering the study. Serum and peritoneal fluid samples were obtained from 60 women in the reproductive age (20-45 y) who underwent laparoscopy from December 2015 to September 2017 in Mother and Child hospital, Shiraz, Iran. The patients did not receive any anti-metabolism and immune-suppressive medication, and hormonal therapy from 3 months prior to laparoscopy. The subjects had no history of either rheumatologic diseases or malignancy in their medical record. Participating women were divided into two groups. Group I included 30 infertile women with late endometriosis (stage III and IV) according to the revised American Society for Reproductive Medicine (rASRM) classification. They had usual unprotected intercourse for at least one year and did not have any other major causes for infertility according to spermogram, pelvic sonography, and hormonal assays (FSH, TSH, PRL). Confirmation and staging of endometriosis for all of patients were determined by the same surgical team, including at least two expert gynecologists. The diagnosis was ultimately certified by histopathologic report. Group II included 30 fertile women with at least two children, and who had undergone laparoscopy for legal tubal ligation, myomectomy, or laparoscopic hysterectomy due to nonstop hemorrhage, with no endometriotic foci, pelvic inflammation, or immunologic pathology observed during laparoscopy.

Sample Collection. Prior to the induction of general anesthesia, 5 mL of peripheral venous blood (PB) was aspirated and transferred into a sterile silicon tube. Following the insertion of trocars into the abdomen and prior to any other manipulation, 2 to 10 mL of peritoneal fluid (PF) was aspirated from cul-de-sac (the space behind the uterus) and treated with heparin. All bloody samples were excluded from the study. Both PB and PF samples were transferred to the laboratory on ice. The peripheral blood and peritoneal fluid samples were centrifuged at 2000 rpm for 7 min. Separated serum and supernatant from PF samples were stored at -40°C until thawing for cytokine measurements.

Cytokine Assay. Concentration of cytokines related to major subsets of T cells (TNF-α, IFN-γ, IL-4, IL-10, IL-23, IL-17, and TGF-β1) in PB and PF were measured by standard cytokine specific Enzyme-linked Immunosorbent Assay (ELISA) using commercial kits (ELISA Ready-Set-Go, eBioscience, affymetrix, Thermofisher, San Diego, USA). The samples were thawed just prior to performing the tests. The sensitivity of the ELISA kits regarding IL-4, IL-10, IL-17, IL-23, TNF-α, IFN-γ, TGF-β1 were 2, 2, 4, 15, 4, 4, 8 pg/mL, respectively.

Statistical Analysis. Statistical analyses (Descriptive, Normality, Mann-Whitney U test and Pearson's correlations) were done using Microsoft Excel and IBM SPSS software Version 18.0. Probability values were calculated on the basis of two-tailed test. All differences in cytokine levels were analyzed by non-parametric independent samples Mann-Whitney U-test. The association between each cytokine and others ones in serum and in PF samples, and also between serum and PF samples were evaluated by Pearson's correlation test.

RESULTS

The present study was performed on 30 infertile endometriosis and 30 non-endometriosis fertile women. The levels of IL-4, IL-10, IL 17, IL-23, TNF-α, IFN-γ, and TGF-β1 cytokines were investigated in both serum and peritoneal fluid samples, and compared between the studied groups. Table 1 indicates the biometry data of the study groups. The mean of age in endometriosis and non-endometriosis women were 31.0 ± 0.9 and 41.1 ± 1 years, respectively. The mean of weight and BMI were 65.5 ± 2.3 and 25.1 ± 0.7 in endometriosis patients, and 70.6 ± 1.8 and 27.8 ± 0.6 in non-endometriosis group, respectively. In this study, the weight and BMI of endometriosis group were lower compared with non-endometriosis group.

Table 1. Demographic Statistics of Study Groups.

	Endometriosis group Mean (SEM)	Non-endometriosis group Mean (SEM)	p-value
Weight (kg)	65.5 (2.3)	70.6 (1.8)	0.010
BMI (kg/m^2)	25.1 (0.7)	27.8 (0.6)	0.010
Age (yr)	31.0 (0.9)	41.1 (1.0)	<0.001

Non-endometriosis group contains women without endometriosis that underwent laparoscopy for TL, myoma, nonstop uterine hemorrhage, Distribution of data was not normal therefore p-value was attained by non-parametric Mann-Whitney test, BMI =Body Mass Index, There was not any selection criterion regarding age and weight.

The concentration of cytokines within serum.

Table 2 represents the results of cytokine assays in the sera of both endometriosis and non-endometriosis groups. As shown, there were no significant differences between two studied groups regarding the concentration of IL-10, IL-17, TNF-α, and TGF-β1 cytokines. Although the difference was not statistically significant concerning IL-23, the calculated p value was close to significance border ($p<0.09$, Table 2). Interestingly, the mean level of TNF-α in endometriosis group was three times higher than non-endometriosis group, an increase not statistically significant due to the high SEM. The level of IFN-γ was significantly lower in endometriosis group as compared with non-endometriosis women ($p<0.001$, Table 2). It is of note that IL-4 was non-detectable in either serum or PF samples. There were significant positive correlations between the serum levels of IL-17 and IL-23, and IL-17 and TNF-α in both endometriosis and non-endometriosis women. Regarding IL-23 and IFN-γ, a positive correlation was detected only in endometriosis group (Table 3).

The concentration of cytokines within the peritoneal fluid.

Statistical analysis indicated that TGF-β1 was significantly increased in the PF of endometriosis group ($p<0.03$, Table 4). Further increases were observed in the levels of IL-17 and TNF-α cytokines in endometriosis women with p values near significance level ($p=0.06$ and $p=0.09$, respectively; Table 4). Regarding IL-10, IL-23 and IFN-γ, there were no significant differences between the two studied groups. There were significant positive

Table 2. Level of Serum Cytokines in Study Groups.

Serum cytokine (pg/ml)	Endometriosis group Mean (SEM)	Non-endometriosis group Mean (SEM)	p-value
IL-10	6.1 (2.8)	10.3 (3.9)	0.160
IL-17	7.2 (0.5)	6.5 (0.2)	0.780
IL-23	26.7 (1.2)	29.6 (1.7)	0.090
TNF-α	37.7 (13.9)	12.9 (4.6)	0.280
IFN-γ	43.2 (10.3)	48.1 (5.2)	<0.007
TGF-β1	7881.8 (445.6)	6852.3 (648.1)	0.530

p-values are calculated by independent samples Mann-Whitney U-test.

correlations between the PF levels of IL-10 and TNF-α, IFN-γ, and TGF-β1 in endometriosis group (Table 5). A strong positive correlation was also observed between IL-17 and IFN-γ in endometriosis group (Table 5). Finally, similar to the serum, there existed a significant correlation among the levels of IL-17 and IL-23 in PF samples from both groups (Table 3, 5).

Table 3. Significant correlations between serum cytokines levels.

Cytokine	IL-23	TNF-α
Endometriosis Group		
IL-17	0.377* p=0.040	0.670** p<0.001
IFN-γ	0.531** p=0.003	
Non-endometriosis Group		
IL-17	0.625** p<0.001	0.457* p=0.014

*Correlation is significant at the level of 0.05 (2-tailed). **Correlation is significant at the level of 0.01 (2-tailed).

Relationship between serum and peritoneal fluid cytokines.

In endometriosis patients, significant positive correlations were observed between the concentrations of different cytokines in the serum and peritoneal fluid, including serum IL-17 and peritoneal IL-17, TNF-α, IFN-γ; serum IL-23 and peritoneal IL-17, IL-23, IFN-γ; serum IFN-γ and peritoneal IL23, IFN-γ; and serum and peritoneal TNF-α (Table 6). In non-endometriosis group, a significant positive correlation was seen only with regards to TNF-α in serum and PF (Table 6). A negative association was further observed between serum IL-10 and PF TGF-β1 in endometriosis patients (Table 6).

Table 4. Level of PF Cytokines in Study Groups.

PF cytokine (picogram/milliliter)	Endometriosis Group Mean (SEM)	Non-endometriosis Group Mean (SEM)	p-value
IL-10	5.5 (0.9)	14.1 (3.5)	0.130
IL-17	4.5 (0.8)	2.6 (0.3)	0.060
IL-23	30.2 (7.1)	22.1 (3.7)	0.750
TNF-α	29.1 (12.7)	14.2 (4.9)	0.090
IFN-γ	7.1 (1.9)	6.9 (1.0)	0.350
TGF-β1	498.0 (8.1)	470.4 (9.3)	0.030

PF= Peritoneal Fluid, p-values are calculated by independent samples Mann-Whitney U-test.

Ratios of serum to peritoneal fluid cytokines.

Evaluation of the serum to PF cytokine ratios and comparison of the ratios of the two study groups showed that the fractions of anti-inflammatory cytokines (IL-10S/IL10P, TGF-β1S/TGF-β1P) were higher, while those of inflammatory cytokines (IL-17S/IL17P, IL-23S/IL23P, IFN-γS/IFN-γP) were lower in endometriosis patients in comparison to non-endometriosis group. However, none of the above differences were statistically significant (Figure 1). Regarding the ratios, Figure 1 shows that inflammatory cytokines in the PF of endometriosis group were increased (except for TNF-α) and PF anti-inflammatory cytokines were reduced. The ratio of TNF-α in endometriosis patients differs from the ratios of other inflammatory cytokines due to the high serum portion of TNF-α in endometriosis patients.

Table 5. Significant correlations between PF cytokines levels.

Cytokine	IL-23	TNF-α	IFN-γ	TGF-β1
		Endometriosis Group		
IL-10		0.430* p=0.018	0.382* p=0.037	0.491** p=0.006
IL-17	0.443* p=0.014		0.741** p<0.001	
		Non-endometriosis Group		
IL-17	0.627** p<0.001			

*Correlation is significant at the level of 0.05 (2-tailed). **Correlation is significant at the level of 0.01 (2-tailed).

It is well known that cytokines act in a network manner and have positive or negative effects on one another. Therefore, what follows is the calculation of the ratios of inflammatory to anti-inflammatory cytokines, and the ultimate summation of inflammatory to anti-inflammatory and Th1 to Th17 inflammatory cytokines in serum

Table 6. Significant correlations between serum and PF cytokine concentrations.

Cytokine	IL-10S	IL-17S	IL-23S	TNF-αS	IFN-γS
		Endometriosis Group			
IL-10P		0.518** p=0.003		0.580** p=0.001	
IL-17P		0.450* p=0.013	0.714** p=0.000		
IL-23P			0.396* p=0.030		0.377* p=0.040
TNF-αP		0.549** p=0.002		0.835** p=0.000	
IFN-γP		0.526** p=0.003	0.729** p=0.000		0.426* p=0.019
TGF-β1P	-0.447* p=0.013				
		Non-endometriosis Group			
TNF-αP				0.566** p=0.001	

*Correlation is significant at the level of 0.05 (2-tailed). **Correlation is significant at the level of 0.01 (2-tailed). S=Serum, P =Peritoneal Fluid.

and PF of endometriosis patients, which was further compared with that of non-endometriosis group. The results indicated that ratios of IFN-γ/TGF-β1 and IL-17/IL-23 were significantly different between endometriosis and non-endometriosis women concerning serum samples ($p<0.001$ and $p<0.01$ respectively, Table 7). Moreover, the ratios of TNF-α/IL-10 and IL-17/IL-10 showed significant differences in the PF samples between the two studied groups ($p<0.04$ and $p<0.03$ respectively, Table 7).

Table 7. Significant Ratios of Cytokines in Serum and Peritoneal Fluid of both Study Groups.

Ratio of cytokines	Serum				p-value	Peritoneal Fluid				p-value
	endometriosis		Non-endometriosis			endometriosis		Non-endometriosis		
	Mean ± SEM		Mean ± SEM			Mean ±SEM		Mean ±SEM		
IFN-γ /TGF-β1	0.007	0.002	0.01	0.005	p<0.001	0.01	0.003	0.01	0.002	0.25
TNF-α /IL-10	18.74	6.54	6.31	2.30	0.14	5.81	1.51	5.40	1.70	p<0.04
IL-17 /IL-10	3.72	0.37	3.16	0.46	0.19	2.50	0.77	2.41	1.07	p<0.03
IL-17 /IL-23	0.27	0.018	0.23	0.01	p<0.01	0.70	0.32	0.36	0.14	0.21

DISCUSSION

In the present study, we investigated the concentrations of a group of cytokines related to T helper subsets within both serum and PF samples to find the importance of these cytokines in the context of endometriosis. Regarding biometry data, the endometriosis

group was significantly younger than non-endometriosis group with lower weights and BMIs, which is logical considering the previous reports as women diagnosed with endometriosis are more often than not younger, taller, thinner, and with significantly lower BMI (3,4,24).

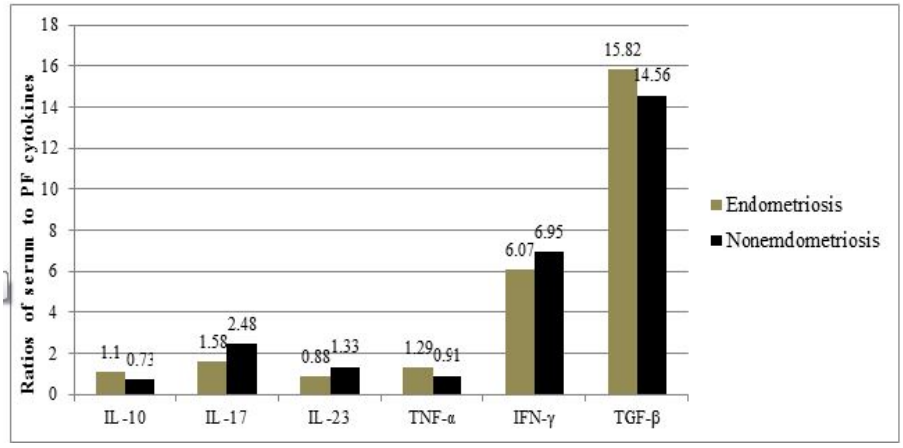

Figure 1. Serum to PF Cytokines in Study Groups. Light columns show serum to PF ratio of cytokines in endometriosis patients and dark columns show this ratio in non-endometriosis group. Analysis was done by use of Mann-Whitney test. PF= Peritoneal Fluid.

In the non-endometriosis group, certain cases sought legal tubal ligation to prevent pregnancy after completing family. On the other hand, some cases of non-endometriosis group were hysterectomized due to nonstop hemorrhage which is more common in older ages. However, non-inflammatory state cannot be claimed for some cases in non-endometriosis group; the reported results in the present study indicated that this limitation did not affect the results because subjects were in the reproductive age, and some inflammatory cytokines showed even lower concentrations in the foregoing group. In general, the results of the present study indicated that there is a significant statistical bias toward inflammatory responses within both serum and PF samples in women complicated with endometriosis. In line with the inflammatory basis in patient group, IL-17/IL-23 in serum, and TNF-α/IL-10, and IL-17/IL-10 ratios in PF samples showed elevated levels in patients. Consistent with the most published data, we found that the level of TNF-α was increased 3 times (although with p=0.09) in the sera of women with endometriosis (11,25). TNF-α/IL-10 was further augmented in patients. TNF-α is secreted mainly from activated macrophages, and promotes the production of other pro-inflammatory cytokines, such as IL-1β and IL-6. Moreover, Th1 cells secrete TNF-α following activation and potentiate inflammation. In addition to affecting the reproductive system, TNF-α plays yet another role in infertility by its toxic effect on embryo (13). There are several reports regarding the relationship between the concentration of TNF-α and disease severity (26,27). The result of the present study regarding the reduced level of IL-10 in endometriosis women was not in agreement with some of the previous reports (1). However, Sipak-Szmigiel and coworkers reported that higher levels of IL-10 might be a discriminating marker between malignancy and endometriosis. In line with the present study, they reported lower levels of IL-10 in endometriosis women with no malignancy

(28). Moreover, the increase in TNF-α/IL-10 ratio in PF samples, reported in the present study, is in line with the idea of inflammatory basis of endometriosis. Interestingly, higher levels of IL-10 have been reported in the early stages of endometriosis (29), while in the late stages of the disease, reduced levels have been revealed (12,30). Based on the published data and the data of the present study, we suggest that IL-10 plays a role at the beginning of the disease, while in the later stages, TGF-β1 predominantly limits the disease progression. Probably corroborating this assumption is significantly higher levels of TGF-β1 in PF samples and the negative significant correlation between serum IL-10 and peritoneal TGF-β1 in endometriosis women in the present research. However, in line with most previous studies, we did not find any relationship between the level of IL-17 and endometriosis (17), but IL-17/IL-10 and IL-17/IL-23 ratios were respectively increased in PF and serum samples from endometriosis group. Elevated levels of IL-17 have been reported in the early stages of the disease (31). In our cases, selected from patients with stages III and IV, the difference between the studied groups concerning -17 was close to significance level ($p=0.06$). Moreover, the increased IL-17/IL-23 ratio reported in patients indicates that the higher level of IL-17 inducer (IL-23) is able to promote more development in Th17 cells as compared with non-endometriosis women. Accordingly, IL-17 might be more important in the initiation, but not in the later process, of endometriosis. Moreover, as expected, IL-17/IL-10 or IL-17/IL-23 ratios reported in the present study might be more informative.

Our results regarding the concentration of IFN-γ within PF samples is also in line with some of the previous reports (32). However, conflicting reports are published regarding the concentration of this cytokine in serum, and the difference between endometriosis and non-endometriosis cases (10,33). In this regard, a group of researchers found a relationship between the concentration of IFN-γ and staging of the disease (34), which other researchers rejected (35). IFN-γ, as the signature cytokine of Th1 cells, is secreted mainly by Th1, NK cells, and activated macrophages. Even though we reported reduced levels of IFN-γ in the peripheral blood samples from endometriosis women, it must be noted that the macrophages are major sources of these cytokines, and the investigation of IFN-γ level in the tissue might be more informative. Our results indicated that IFN-γ is decreased in the serum samples from women complicated with endometriosis, and IFN-γ/TGB-β1 ratio also significantly showed down-regulation in the patients. TGF-β1 supports both Th17 and Treg cells differentiation in a dose dependent manner. In higher concentrations, the immune response will shift toward Treg cells (36). Therefore, higher concentrations of TGF-β1 might regulate the inflammation in the patients via reducing IL-17 in the later stages of endometriosis, which is in line with the absence of a significant difference in IL-17 level in our study. On the other hand, TGF-β1 might stimulate macrophages and fibroblasts to synthesize collagen and matrix modifying enzymes to repair inflamed tissues. This mechanism results in the formation of fibrotic attachment among organs in the peritoneal cavity of endometriosis patients, especially in late stages, which is in line with the increased concentration of TGF-β1 reported in the present study. Although there is controversy regarding the level of TGF-β1 in serum or PF samples, almost all published papers have underscored the over-expression of this cytokine in both sera and PF samples from women complicated with endometriosis (37). TGF-β isoforms, sensitivity of the kits and sampling methods are the most important reasons behind such controversies. In our study, IL-23 level did not significantly change in the serum and PF of late endometriosis group compared to non-endometriosis women. There are several reports regarding the increase in the levels of IL-23 in the PF samples of women in early

endometriosis stages, but not in the serum (17). Previous studies have also shown that IL-17 and IL-23 were more important in inflammation mediated by neutrophils in the early phases of the disease (5,38). In accordance with these data, the present study reported that IL-17/IL-23 ratio was increased in the periphery of endometriosis women. Based on the results of the present study, the correlations between IL-23 and IL-17 may further confirm the cooperation of these cytokines in the context of the disease. The analysis of the correlation between cytokine levels indicated that there was a complex network controlling the immune responses in endometriosis. Several critical points must be considered regarding the role of cytokines' network in the context of the disease. Conflicting results might be due to the different ethnic group populations, variety of the disease nature, sample type and sampling time, stage of the disease, and technical issues in sampling and performing the tests. Moreover, the level of a same cytokine might be reported to be increased in one sample (for example serum) while reduced in another (for example PF). We hypothesize that in every menstrual cycle, T cell subsets (Th17, Th1, Th2, and Treg cells) play roles in increasing and braking inflammation. In the beginning, due to the absence of adequate defense mechanism and by the signals of the innate immunity, Th17 cells start inflammation which is followed by Th1 responses. Gradually, after hormonal changes, Th2 cells might become dominant and change the balance towards the activation of regulatory T cells and tissue repair. This scenario is repeated monthly in every menstrual cycle. Fibrosis, severe organ attachments and endometriosis development occur after several years.

In conclusion, endometriosis is not a stage performance merely belonging to one of the T cell subsets, and it seems that cytokines play their specific roles. While the ratios of inflammatory cytokines such as IFN-γ, TNF-α, and IL-17 increase in infertile endometriosis women, they are antagonized by anti-inflammatory cytokines such as IL-10 and TGF-β1.

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