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Research Article

Different Expressions of T-helper Cell Subsets-Related Transcription Factors and Cytokines in Infertile Women with Endometriosis

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Abstract

Purpose: It is suggested that alterations in immunologic mechanisms intervenes in the pathophysiology of endometriosis and the disease complications. There are several published reports regarding alteration in T helper cell subsets in the pathology of reproductive organs. This study was designed to find the pattern of T helper subsets in endometriosis compared with non-endometriosis women.

Methods: Expression of T helper subset-specific transcription factors (*TBET*, *GATA3*, *RORC*, *FOXP3*) and related cytokines, including *IL-4*, *IL-10*, *IL-17*, *IL-23*, *TNFA*, *IFNG* and *TGFB* were investigated by real time-PCR in peripheral blood, peritoneal fluid, eutopic and ectopic endometrium.

Results: The expressions of *RORC*, *FOXP3* and all the studied cytokines were significantly increased in blood mononuclear cells of endometriosis group. The expression levels of *TBET*, *FOXP3*, *IL-17A* and *TGFB* were significantly elevated in peritoneal fluid mononuclear cells of endometriosis women. Besides, the expression levels of *GATA3*, *RORC*, *FOXP3*, *IL-4*, *TNFA*, and *IFNG* were significantly increased in eutopic endometrium of endometriosis patients. Moreover, *GATA3*, *FOXP3* and *IL-4* showed increased expressions in ectopic endometrium, while *TBET*, *IL-23* and *IFNG* were significantly decreased in comparison with endometrium of non-endometriosis women. Finally, the expression levels of *TBET*, *RORC*, *IL-23*, *TNFA*, and *IFNG* significantly declined in ectopic endometrium in comparison with eutopic endometrium among endometriosis women.

Conclusions: Based on the present study results, it seems Th17, Th1, Th2, Treg cells and their related cytokines may play influencing role in progression of endometriosis and its symptoms including infertility. Indeed, cytokine network imbalance may cause the disease progression in patients during several years.

Keywords: Endometriosis; Infertility; T cell subsets; Transcriptional Factors

Abbreviations

PB: Peripheral Blood; PF: Peritoneal Fluid; Th: T helper; Treg: T regulatory; IL: Interleukine; IFN: Interferone; TGF: Transforming Growth Factor; TF: Transcriptional Factor; TNF: Tumor Necrotizing Factor; PBMC: Peripheral Blood Mononuclear Cell; PFMC: Peritoneal Fluid Mononuclear Cell; Eeu: eutopic Endometrium; Eec: ectopic Endometrium; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; PRL: Prolactin; TSH: Thyroid Stimulating Hormone

Introduction

Endometriosis is a common chronic inflammatory pelvic disease in women at reproductive age with two main manifestations, including pain and infertility [1,2]. This disease is defined as the presence of endometrial tissue out of the uterine cavity [2]. Although the exact etiology of endometriosis is not yet well known, the most accepted theory is retrograde menstruation, which could initiate immune system responses to ectopically implanted endometrial cells on the peritoneal lining among 10 to 15% of women [3,4]. It is suggested that alterations in immunologic mechanisms intervenes in the pathophysiology of endometriosis and the disease complications [3]. In women with endometriosis, significant changes have been reported in population of immune cell subsets and types of responses in the Peripheral Blood (PB), Peritoneal Fluid (PF) as well as endometrial tissues [5,6]. Various leukocytes reside in eutopic endometrium and also endometriotic implants, and produce different cytokines and other immune mediators [5,7]. There are several reports regarding deviation of Th cells responses in endometriosis [8-10]. While predominance of Th1 cells may promote inflammation, increase in Th2 cells modulates inflammation in affected areas. Th1 cells potentiate inflammation by producing IFN-γ. Depending on the level of secretion, IFN-γ and TNF-α are proposed to have dual effects on early stage of gestation and implantation [11]. On the other hand TNF-α has toxicity on sperm and egg cells and activates Th1cells [12]. TNF-α may also be produced by Th1. Th2 cells secrete IL-4, IL-10, and TGF-β as anti-inflammatory cytokines. Increase of IL-4 and IL-10 is reported in some research on endometriosis [10]. In addition, Th17 and regulatory T (Treg) cell subsets have been also proposed

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to play roles in pathogenesis of endometriosis [13-15]. Th17 cells may trigger inflammation by production of neutrophil attractive chemokines [16]. It is argued that Th17 cells deal with induction of many autoimmune diseases, including arthritis, inflammatory bowel disease, psoriasis, uveitis, and multiple sclerosis [17,18]. IL-17A is the most important cytokine produced by Th17 cells, which mediates neutrophilic inflammation [19].

Increase in Treg cells inhibits the recruitment of effector immune cells. Treg cells also produce anti-inflammatory IL-10 and TGF-β cytokines [4]. It is reported that women with endometriosis show decreased level of cytokines IL-17 and TGF-β some weeks after laparoscopy [20]. This is also followed by increase in pregnancy rate during next months.

Moreover, it is well documented that Th cells and their related cytokines cooperate with each other as a network. So, disturbance in Th subsets could be accounted as a major immunological etiology for many infertility-related diseases including endometriosis [21,22]. In line with this idea, several studies have shown that the proportion of immune cells and cytokine production in women with endometriosis has altered in comparison with healthy women [23,24]. In addition to signature cytokines, investigation of Th subset-specific Transcription Factors (TFs) has been also conducted with the aim of evaluating the Th balance in endometriosis [25,26].

Interestingly, there is no published data regarding the simultaneous investigation and comparison of the Th subsets balance in all the potential samples, including PB, PF, intra (eutopic), and extra (ectopic) uterine endometrial tissue samples in endometriosis patients. In the previous published research, only one or two source of PB, PF, eutopic or ectopic uterine endometrial tissues have been investigated. So, in the present study, the expression of TFs and cytokines related to Th1, Th2, Th17, and Treg in PB, PF, and endometrial tissues were simultaneously investigated to elucidate the pattern and the role of T cell subsets involved in endometriosis pathogenesis. For this purpose, we analyzed and compared genes expression of *TBET*, *GATA3*, *RORC*, *FOXP3*, *IL-4*, *IL-10*, *IL-17*, *IL-23*, *TNFA*, *IFNG*, and *TGFB* from endometriosis and healthy women in all the mentioned samples.

Materials and Methods

Subjects

This 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 written informed consent before entering this research study and sampling. Clinical and demographic features of the participants are shown in Table 1.

In this study, two groups of women were compared. Both groups were at reproductive age (20-45 years old) and had not received either any anti-metabolism and immune-suppressive medications or hormonal therapy three months prior to laparoscopy. Based on the medical records, the subjects had no history of autoimmune diseases or malignancy. Group I included 30 infertile women with late-stage endometriosis.

Infertile late endometriosis group consisted of patients with stages III and IV according to the revised American Society for Reproductive Medicine (rASRM) classification. Confirmation and staging of endometriosis were determined through laparoscopy by the same surgical team including two expert gynecologic laparoscopists. The patients have had usual unprotected intercourse for at least one year without pregnancy and had no other causes for infertility on the basis of spermogram, pelvic sonography, and hormonal assays (FSH, LH, TSH, and PRL). In this study group, peripheral blood, peritoneal fluid, eutopic (Eeu), and ectopic (Eec) endometrial tissue samples were obtained. Group II included 30 fertile women as the fertile non-endometriosis. This control group consisted of women, who had at least two children and underwent laparoscopy due to legal tubal ligation, myomectomy, or nonstop bleeding hysterectomy. In this group, no endometriotic foci or immunologic pathology were observed during laparoscopy. The obtained samples from fertile non-endometriosis group were similar to endometriosis group except lack of ectopic endometrium.

Table 1: Clinical and demographic features of women participated in this study.

	E	NE
Reproductive age	Yes	Yes
Fertility	No	Yes
Cause of laparoscopy	Clinical endometriosis	Tuba ligation, hemorrhage, Myoma
Confirmed endometriosis	Yes	No
Known autoimmune diseases	No	No
Malignancy	No	No
Hormone therapy	No	No
Samples	PB, PF, Eeu, Eec	PB, PF, Eeu

E: Endometriosis; NE: Non-Endometriosis; PB: Peripheral Blood; PF: Peritoneal Fluid; Eeu: Eutopic Endometrium; Eec: Ectopic Endometrium.

Sample collection

Women who had the described criteria of one of the study group in their records and were taken to operating room for laparoscopy were put in the appropriate list. Before general anesthesia, 10mL of peripheral venous blood was aspirated by a heparinized syringe and transferred into a sterile tube. After induction of anesthesia and insertion of trocars and before any other manipulation, 2 to 10 mL of peritoneal fluid was aspirated from Douglass pouch and treated with heparin. All the bloody samples were excluded from the study. Mononuclear cells of both PB (PBMCs) and PF (PFMCs) were collected using Ficoll hypaque and density gradient centrifugation at 3000 rpm for 20 minutes. The aliquots containing two million cells were stored at -70°C until RNA extraction. Moreover, extra-uterine endometrial tissue samples of pelvic endometrium were obtained from endometriosis patients. One part of these tissue samples was laid in formalin for the ultimate histopathological confirmation of the disease, and the other part was placed in physiological serum for RNA extraction. Finally, eutopic endometrial tissue samples were collected by curettage from both groups. All these tissue samples were washed adequately to remove blood and stored at -70°C until RNA extraction.

Quantitative real-time PCR (qRT-PCR)

To reach an accurate result in real time-PCR, items of MIQE guidelines were considered. Total RNA from PBMCs, PFMCs, eutopic, and ectopic endometrial tissue samples were extracted by

Table 2: Sequences of primers used for qRT-PCR.

Gene	Forward Primer	Reverse Primer
TBET	GGATGCGCCAGGAAGTTTCA	GACTGGAGCACAATCATCTGGG
GATA3	AGCACAGAAGGCAGGGAGTGT	TGATAGAGCCCGCAGGCG
FOXP3	GAGAAGGGCAGGGCACAAT	TGGGCCTGCATGGCAC
RORC	GGCAAATACGGTGGCATGG	AAGGCACTTAGGGAGTGGGAGA
TNFA	GCCTGCTGCACTTTGGAGTG	TCGGGGTTCGAGAAGATGAT
IFNG	GTGTGGAGACCATCAAGGAAGACA	TTGGACATTCAAGTCAGTTACC
TGFB	AAATTGAGGGCTTTCGCCTTA	TGAACCCGTTGATGTCCACTT
IL-4	TCCGATTCCTGAAACGGCT	TCTGGTTGGCTTCCTTCACAG
IL-10	GCCTAACATGCTTCGAGATC	TGATGTCTGGGTCTTGGTTC
IL-17	CAATGACCTGGAAATACCCAA	TGAAGGCATGTGAAATCGAGA
IL-23	GGACAACAGTCAGTTCTGCTTGC	AGGCTCCCCTGTGAAAATATCG
RPL13a	CATAGGAAGCTGGGAGCAAG	GCCCTCCAATCAGTCTTCTG
ACTB	TGTGATGGTGGGTATGGGTC	ACACGCAGCTCATTGTA

using a commercial Total RNA extraction kit (Parstous, Mashhad, Iran) according to the manufacturer instruction. Possible genomic DNA was removed by treating RNA samples with RNase-free DNase I (Thermo Fisher Scientific, Carlsbad, USA). cDNA was also synthesized using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Carlsbad, USA). Using Genevestigator biomedical software, *RPL13a* and *ACTB* were determined as reference genes for mononuclear cells and endometrial tissue samples, respectively. Primer sequences were selected by means of Allel ID 7.5 software and primer-BLAST (Table 2). Furthermore, real time-PCR amplifications were performed using Applied Biosystems SYBR Green (Thermo Fisher Scientific, Carlsbad, USA) and ABI 7500 real time-PCR system (Applied Biosystems, Foster City, California, USA). Real time-PCR amplification programs were as follows: 95°C (10′) 94°C (30″) 60°C (1′) for *TBET*, *GATA3*, *FOXP3*, *TNFA*, *TGFB*, IL-4 and 95°C (10′) 95°C (15″) 60°C (30″) 72°C (30″) for IL-10, IL-17, *IL-23*, *RORC*, *IFNG*, *RPL13a*, *ACTB*.

Statistical analysis

Statistical analyses were performed using IBM SPSS software Version 18. Normality of the data was assessed by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Probability values were calculated on the basis of two-tailed tests. All differences in fold changes were analyzed by the non-parametric Kruskal-Wallis test (pot-hoc: Mann-Whitney U-test). The correlations between the expression levels of different studied genes were calculated by Pearson's correlation test.

Fold Change, ΔΔCt, and ΔCt were calculated by the following formula in which E and NE are representatives of endometriosis and non-endometriosis groups respectively.

$\Delta Ct_E = Ct_E$ (target gene) - Ct_E (reference gene)

$\Delta Ct_{NE} = Ct_{NE}$ (target gene) - Ct_{NE} (reference gene)

$\Delta\Delta Ct = \Delta CtE - \Delta CtNE$

Fold Change = $2^{-\Delta\Delta Ct}$

Results

Expression of the T helper cells transcription factors

Expression of TFs in PBMCs and PFMCs: Figure 1 and 2 indicate the results of expression of Th cells' TFs between endometriosis and non-endometriosis groups using PBMCs and PFMC samples. As shown in Figure 1, the expression levels of *RORC* and *FOXP3* in PBMC samples were significantly increased in endometriosis group compared with the non-endometriosis group (P=0.0001 for both TFs).

Regarding PFMCs samples in Figure 2, *TBET* and *FOXP3* have significantly over-expressed in endometriosis group (P=0.001 and P=0.0001, respectively).

We, additionally, analyzed the ratios between the expression of the studied inflammatory and anti-inflammatory transcription factors in both PBMC and PFMC samples (Table 3). Within PBMC samples, the ratios of *TBET* Δct/*GATA3* Δct, and *TBET* Δct/*FOXP3* Δct were significantly higher in endometriosis in comparison with non-endometriosis group (P=0.004 and P=0.0001, respectively). Regarding PFMC samples, *TBET* Δct/*GATA3* Δct ratio was significantly decreased in endometriosis group (P=0.0001). However, the ratio between sum of inflammatory and sum of anti-inflammatory TFs (Δct *TBET* + Δct *RORC*/Δct *GATA3* + Δct *FOXP3*) was significantly lower in endometriosis women in both PBMC and PFMC samples (P=0.0001 for both comparisons).

Also, in terms of PBMCs in the control group, there were significant positive correlations between Δct of *TBET* and two TFs *GATA3* and *FOXP3* (P=0.0001 and P=0.003 respectively), between *GATA3* and *FOXP3* (P=0.001), and between *FOXP3* and *RORC* (P=0.0001, Table 1 in the supplemental file). All the TFs were also significantly correlated with each other in PFMC of this group (P=0.0001 for all the correlations, Table 1 in the supplemental file). On the other hand, in PBMC of endometriosis patients, there was only significant positive correlation between *GATA3* and *FOXP3* (P=0.012). In PFMCs of this group, however, all the TFs were significantly correlated with each other (P=0.0001 except between *TBET* and *RORC* with P=0.006, Table 2 in the supplemental file).

Expression of TFs in endometrial tissues: Results of the

Table 3: The ratios of inflammatory to anti-inflammatory TFs in PBMC and PFMC of endometriosis and non-endometriosis groups.

Ratios of TF Δct	PBMC			PFMC		
	NE	E	*P* Value	NE	E	*P* Value
	Mean(±SEM)	Mean(±SEM)		Mean(±SEM)	Mean(±SEM)	
TBET Δct/*GATA3* Δct	0.33(0.05)	0.57(0.05)	P=0.004	0.96(0.06)	0.76(0.05)	P=0.0001
TBET Δct/*FOXP3* Δct	0.36(0.09)	1.06(0.71)	P=0.0001	0.98(0.03)	0.90(0.18)	P=0.060
TBET Δct +*RORC* Δct/*GATA3* Δct + *FOXP3* Δct	12.02(0.99)	7.62(0.54)	P=0.0001	18.78(1.11)	13.97(0.74)	P=0.0001

TF: Transcriptional Factor; NE: Non-Endometriosis Women; E: Endometriosis.

Table 4: Ratios of inflammatory to anti-inflammatory TFs Δct and fold change between different endometrial tissues.

Ratios of fold changes of Endometrial tissues TFs	Mean of Ratios of $2^{-\Delta\Delta ct}$ (±SEM)			*P* Value		
	NE	Eeu	Eec	NE *vs.* Eeu	NE *vs.* Eec	Eeu *vs.* Eec
TBET Δct/*GATA3* Δct	5.63(4.68)	0.64 (0.18)	12.56(9.91)	0.293	*P*=0.0001	*P*=0.0001
TBET Δct/*FOXP3* Δct	-0.15(0.55)	14.30(10.70)	2.11 (4.38)	*P*=0.003	*P*=0.0001	*P*=0.046
TBET Δct + *RORC* Δct/*GATA3* Δct + *FOXP3* Δct	-2.23(8.11)	3.26 (0.55)	2.13 (3.43)	*P*=0.001	0.089	*P*=0.013

TFs: Transcriptional Factors; NE: Non-Endometriosis Women; E: Endometriosis.

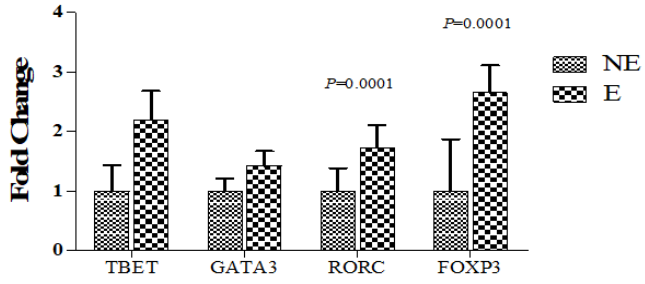

Figure 1: Genes expression of transcription factors in PBMCs of women with endometriosis (E) in comparison with non-endometriosis women (NE).

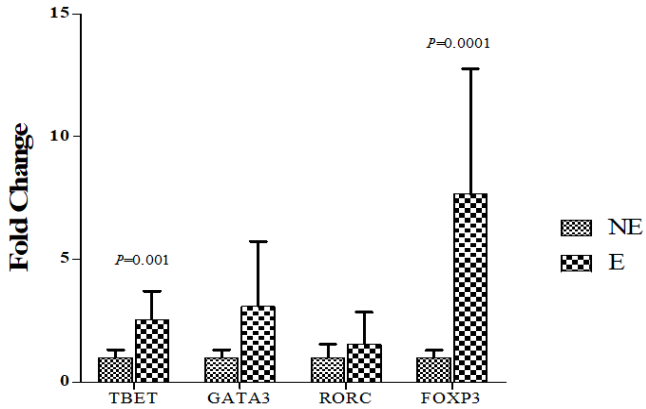

Figure 2: Genes expression of transcription factors in PFMCs of women with endometriosis (E) in comparison with non-endometriosis women (NE).

expression of TFs in endometrial tissues are presented in Figure 3. As indicated, the expression of *GATA3*, *RORC*, and *FOXP3* were significantly increased 2, 1.08, and 2.7 folds, respectively, in eutopic endometrial tissue from endometriosis patients compared with non-endometriosis control group (*P*=0.007, *P*=0.017, *P*=0.0001). Regarding ectopic tissues, the expression of *GATA3* and *FOXP3* were increased 2.38 and 2.76 folds, respectively (*P*=0.015, *P*=0.0001), while *TBET* was significantly down regulated (*P*=0.0001) in women with endometriosis compared with non-endometriosis control group.

When comparing ectopic and eutopic tissues, the expression of inflammatory TFs *TBET* and *RORC* significantly decreased in ectopic tissue of endometriosis patients (*P*=0.0001, *P*=0.037).

Analysis of the ratios of inflammatory to anti-inflammatory TFs and comparison of these ratios in endometrial tissues are presented in Table 4. *TBET* Δct / *GATA3* Δct was significantly higher in ectopic tissue than endometrium of control and endometriosis groups, almost 2.2 and 19.6 times respectively (*P*=0.0001 for both comparisons). Moreover, *TBET* Δct / *FOXP3* Δct significantly increased by over 97

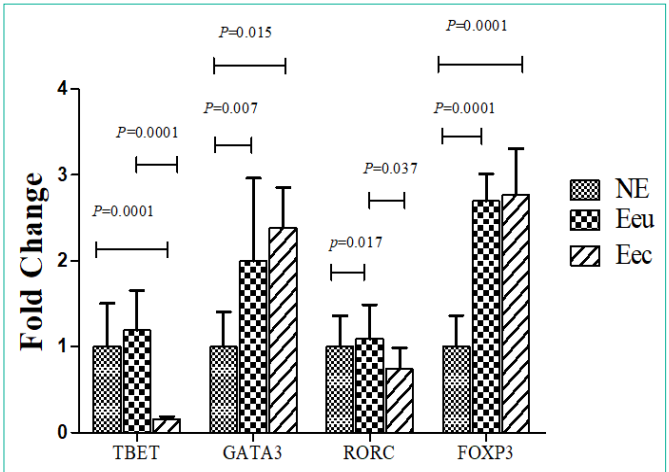

Figure 3: Genes expression of transcription factors in eutopic and ectopic endometrial tissues (Eeu and Eec) of women with endometriosis in comparison with endometrium and non-endometriosis women (NE).

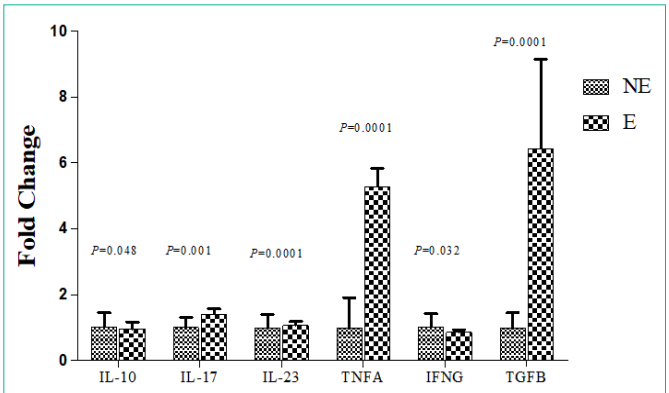

Figure 4: Genes expression of cytokines in PBMC of women with endometriosis (E) in comparison with non-endometriosis women (NE).

times in eutopic endometrium of endometriosis patients compared with the control group (*P*=0.003). Also, *TBET* Δct / *FOXP3* Δct in ectopic endometrial tissue of endometriosis women significantly rose 15 times compared with endometrium of the control group (*P*=0.0001), it significantly declined in comparison with eutopic endometrium of the same group, though (*P*=0.046). Similarly, the ratio between sum of inflammatory TFs expression and the sum of anti-inflammatory TFs was significantly increased in eutopic endometrium of endometriosis women versus endometrium of the control group (*P*=0.001). This ratio indicated decreasing in ectopic endometrial tissues of endometriosis patients in comparison with endometrium of the same group (*P* = 0.013).

Likewise, significant positive correlations were found between

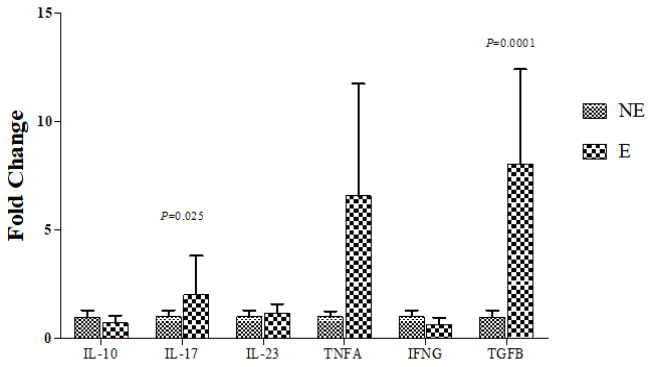

Figure 5: Genes expression of cytokines in PFMC of women with endometriosis (E) in comparison with non-endometriosis women (NE).

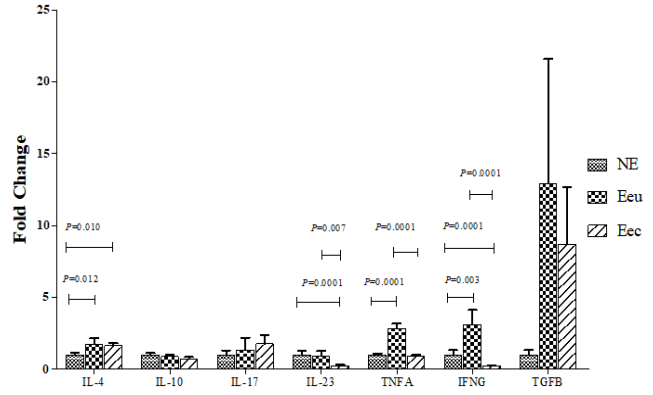

Figure 6: Genes expression of cytokines in eutopic (Eeu) and ectopic (Eec) endometrial tissues of women with endometriosis in comparison with endometrium and non-endometriosis women (NE).

Δct of (*GATA3* and *TBET*), (*GATA3* and *FOXP3*), and (*GATA3* and *RORC*) in endometrium of the control group (*P*=0.006, *P*=0.0001, *P*=0.015, respectively). In eutopic endometrium of endometriosis patients, correlations between (*FOXP3* and *GATA3*) as well as (*FOXP3* and *RORC*) were significantly positive (*P*=0.034, *P*=0.015). In ectopic endometrium of endometriosis patients, only *GATA3* and *RORC* displayed a significant positive correlation (*P*=0.001, Table 3 in the supplemental file).

Difference in the Expression of Cytokines

Cytokines in PBMC and PFMC: Concerning PBMC, Figure 4 shows that the expression of cytokines *IL-17*, *IL-23*, *TNFA*, and *TGFB* significantly increased (*P*=0.001 for IL-17 and *P*=0.0001 for the others) in endometriosis patients, while expressions of *IL-10* and *IFNG* significantly decreased to 0.95 and 0.85, respectively (*P*=0.048, *P*=0.032). On the other hand, considering PFMC in Figure 5, the expression of *IL-17* and *TGFB* significantly increased 2 and 8 times in endometriosis women, respectively (*P*=0.025, *P*=0.0001).

Despite using different *IL-4* primer sets, the product of *IL-4* gene was not detected by qRT-PCR in PBMC and PFMC samples.

Besides, in the control group, there were significant positive correlations between Δct of all cytokines in PBMC (different P values) with two exceptions between *IL-17* and *TNFA* as well as IL-17 and *IFNG*. Considering PFMCs of control women, all cytokines were positively correlated (*P*=0.0001, Table 4 in the supplemental file).

On the other hand, in PBMC of endometriosis women, there were significant positive correlations between cytokines except for *TNFA*. Also, there was no correlation between IL-10 and *IFNG* and between *TGFB* and *IFNG*. In PFMC of this group, significant positive correlations were observed between all cytokines (different P values) except *TNFA* and IL-17 as well as between *TNFA* and *IFNG* (Table 5 in the supplemental file).

Cytokines in endometrial tissues: Results of the investigation of cytokines in endometrial tissues are presented in Figure 6. Expression of IL-4 increased in eutopic and ectopic tissues of endometriosis patients compared with endometrium of the control group (*P*=0.012, *P*=0.010). Expression of *IL-23* decreased in ectopic endometrial tissue of endometriosis women compared to eutopic endometrium of both endometriosis and non-endometriosis women (*P*=0.007, *P*=0.0001, respectively). Regarding *TNFA*, it increased in eutopic endometrium of patients compared with non-endometriosis tissue, but it decreased in ectopic endometrial tissue compared with eutopic of endometriosis patients (*P*=0.0001 for both comparison). Compared with endometrium of the control group, expression of *IFNG* increased 3 times in eutopic endometrium of endometriosis patients (*P*=0.003). On the other hand, this cytokine decreased in ectopic endometrial tissue compared to eutopic endometrium of the same group and also non-endometriosis women (*P*=0.0001, for both).

In endometrium of control group, Δct of IL-4 and *IFNG* had significant positive correlation (*P*=0.006). There was also significant positive correlation between IL-10 and *TGFB* (*P*=0.020).

Regarding eutopic endometrium of endometriosis patients, IL-4 and *TNFA* had significant positive correlations (*P*=0.014). Finally, in ectopic endometrial tissue of the patients, IL-4 and IL-17 were significantly correlated (*P*=0.047). In addition, IL-17 had significant positive correlations with IL-10, *IL-23*, and *TNFA* (*P*=0.029, *P*=0.011, *P*=0.025). Also, we noticed significant positive correlations between *IL-23* and IL-10 as well as between *IL-23* and *TNFA* (*P*=0.007, *P*=0.010, Table 6 in the supplemental file).

Contrarily, there were significant negative correlations between Δct of IL-10 in eutopic endometrium and IL-4, IL-10, and *TGFB* in ectopic endometrial tissue in endometriosis patients (*P*=0.022, *P*=0.042, *P*=0.019 respectively). Also, a significant negative correlation was found between *IFNG* in eutopic and *IL-23* in ectopic endometrial tissues in endometriosis patients (*P*=0.034, Table 7 in the supplemental file).

Discussion

Endometriosis is a hormone-dependent disease in which immune cell populations change especially in four major parts: PB, PF, endometrium, and peritoneal implants. Subpopulations of T cells that reside in these parts determine immune responses and the disease manifestations, such as infertility and pain [27]. Imbalance between Th1 and Th2 cells, and decrease of Treg cells have been suggested for establishment of endometriosis [28].

Transcriptional factors

Considering PBMCs in this study, we found that the elevations

of *RORC* and *FOXP3* were significant. Previously, in accord with our findings, Ahn SH et al. and Hirata T et al. reported increased level of IL-17, the main cytokine of Th17, in the serum of endometriosis patients [20,29]. These findings may highlight the role of Th17 cells in the pathogenesis of endometriosis. However, regarding *FOXP3*, our finding was not consistent with what Olkowska-Truchanowicz et al. found in their study probably because they selected endometrioma patients [15]. Besides, there is no consensus about the frequency of Treg cells or increase of *FOXP3* expression in PB [30].

Regarding PFMCs, in the present study, increases in mRNA expressions of *TBET* and *FOXP3* were significant. In this regard, Ho et al. announced that all subsets had increased in endometriosis group compared with the control group, but Th1 cells had increased less than other subsets [31]. Richter ON et al. and Othman EER et al. also reported increase in Th1 axis cytokines, including TNF-α and IFN-γ [32,33]. As peritoneum is the main area of inflammation, increase of Th1 cells in PF is expectable. Also, the increased level of *FOXP3* in the PF was in accordance with the result of other studies that showed elevated frequency of Treg cells in the PF [15,34]. Some previous research had conflicting results regarding Treg cells of PB and PF between endometriosis and control groups [35]. Logically, as endometrial implanted cell, antigens are in close contact with the active immune cells in the peritoneal lining and fluid, these cells should be more responsive than those in the blood circulation. Therefore, increased frequency of Treg cells might be a regulatory and inhibitory mechanism in pelvic peritoneum.

Furthermore, regarding the correlations between TFs of T cell subsets in PBMCs and PFMCs, a network mechanism was observed. Significant correlations between TFs of PBMCs in healthy women showed that a potent regulated mechanism exists between TFs, but in endometriosis women, only the correlation between *GATA3* and *FOXP3* was significant. This could be explained as the disruption of the cytokine and TFs network mechanism in the patients. Considering PFMC, all the TFs were significantly correlated in both study groups. This might also be the result of close cooperation of different immune cells in the peritoneum.

The results of the present study regarding gene expressions and correlations in PBMCs and PFMCs indicated that both inflammatory and anti-inflammatory subsets are activated. In addition, the similarity of diagrams shows the presence of a systemic immune response even though presentation of inflammation in endometriosis is mainly focused in pelvic cavity.

In this study, comparison of eutopic endometrium of endometriosis patients with the control group revealed significant increases in mRNA expressions of *GATA3*, *FOXP3*, and *RORC*. In accordance with the current study, Podgaec S. et al. and Chen P. et al. found increased expression of *GATA3* and Th2 cytokines in endometriosis [10,36]. So, endometriosis can be considered as an inflammatory disease with Th2 dominance [10]. In addition, in the present study, the increased level of *FOXP3* T cells in endometrial tissues of endometriosis patients is in accordance with findings of Berbic M et al. [37]. However, previous studies presented conflicting results on *FOXP3* gene expression or Treg cell frequency in eutopic endometrium [28,38]. For instance, Jasper et al. reported a decrease of *FOXP3* expression in the secretory phase [25], which is not in consistence with our result, as we investigated *FOXP3* expression in proliferative phase. Also, we found that the expression of *RORC* was higher in endometrium of endometriosis patients in comparison with endometrium of non-endometriosis group. This is in line with the Takamura's study and shows that Th17 cells have a major role in endometriosis [39]. In line with most of the previous studies, we did not find significant increase of *TBET* expression in eutopic endometrium of endometriosis patients. Inman D et al. showed cyclic presentation of *TBET* and *GATA3* in normal endometrium during menstrual cycle; hence, time of sampling as well as causes of infertility in control group can influence on the results [40]. For example, Koval H et al. found an increase in *TBET* and *GATA3*, but they reported decrease in *FOXP3* expression. In this research, they compared endometriosis patient with infertile women with tubal causes [28]. Also, many other studies recognized increases in Th1-related cytokines.

Considering ectopic endometrial tissue, we noticed that the expression of *GATA3* and *FOXP3* significantly increased in comparison with endometrium of the control group, but *TBET* decreased significantly. Takamura et al. also reported decrease of Th1 cells in ectopic endometrial tissue [39]. As the expressions of *TBET* and *GATA3* are regulated in the opposite manner, increase of *GATA3* and decrease of *TBET* are expected observations [41,42]. In line with current study results, Podgaec S et al. and Chen P et al. reported increase of *GATA3*, Th2 cells and their related cytokines in ectopic lesions [26,43]. Regarding *FOXP3*, Budiu et al. reported increased expression of this molecule in the context of endometriosis [44]. Besides, two other experiments reported an increase in the frequency of Treg cells in ectopic endometrial tissues [37,45].

In this study, *TBET* and *RORC* decreased significantly in ectopic endometrial tissue in comparison with eutopic tissue in endometriosis patients. We did not find any papers on *TBET* gene expression in ectopic endometrial tissues, but Takamara et al. found that the proportion of Th1 cells in ectopic lesions had been significantly lower than eutopic endometrium [39]. As to *RORC*, our finding did not support that of Takamura et al. since they found higher Th17 cells in ectopic lesion in comparison with endometrial tissue [39]. Frequency of these cells was also higher in PF of endometriosis patients and in association with the severity of the disease [46]. Then according to the current study, increase of Th17 cells is prominent in PF and not within the lesions. Significant decrease of inflammatory TFs in ectopic lesions seen in this study might be due to suppression by active anti-inflammatory factors.

Some of the previous studies found that the ratio of Th1/Th2 could be more informative than frequency of a subset or expression of a single TF to discriminate the disease [42,47]. Meanwhile, some research reported the imbalance between Th1/Th2 cells in endometriosis. For example, Siedentopf et al. noticed a higher Th1/Th2 ratio in the PB of patients affected by endometriosis [48]. Regarding the ratios of inflammatory to anti-inflammatory TFs (subsets), this study showed that *TBET*/*GATA3* and *TBET*/*FOXP3* ratios were higher in PBMCs of endometriosis patients in comparison with the control group. This certified that inflammation had occurred systemically in endometriosis patients. On the other hand, in PFMCs of these patients, all of the inflammatory to anti-inflammatory ratios decreased in comparison with the control group. These ratios

suggested that severity of inflammation has especially provoked activation of braking cells and more potent anti-inflammatory pathways in PF.

In eutopic endometrium of endometriosis patients in comparison with endometrium of the control group, *TBET*/*FOXP3* as well as the *TBET* + *RORC*/*GATA3* + *FOXP3* ratios significantly decreased. These ratios might suggest that regulatory pathways, especially Treg cells are more active in endometriosis patients. Comparing ectopic endometrial tissue with endometrium of the control group, *TBET*/*GATA3* and *TBET*/*FOXP3* ratios significantly increased. On the other hand, comparison of ectopic and eutopic endometrial tissues revealed that *TBET*/*GATA3* ratio significantly rose in ectopic tissue, but *TBET*/*FOXP3* and *TBET*+*RORC*/*GATA3*+*FOXP3* ratios decreased. These findings might also propose that inflammation is augmented in extra-uterine lesions but potently suppressed by anti-inflammatory factors.

Cytokines

In the present study, moreover, all the investigated cytokines expressed differently in PBMCs of endometriosis patients compared with non-endometriosis women. The mRNA expressions of IL-10 and *IFNG* decreased significantly in endometriosis patients, while mRNA expression of other cytokines increased. The findings about IL-10 mRNA expression is not in accordance with the study by Antsiferova et al. [49], but it is in line with findings of two other experiments that reported decreased IL-10 level in late-stage of endometriosis [50,51]. Likewise, with regard to IFN-γ, Othman et al. reported an increase in this cytokine in PB of endometriosis patients [33], but most of others did not report any difference or decrease of this cytokine [52]. On the other side, in accordance to our results increase in TNF-α serum was reported in many studies. Some of them showed association between TNF-α level and severity of endometriosis [43,53]. The elevated expression of *TGFB* was also in line with Kyama CM's investigation [54]. TGF-β is a cytokine produced by Treg cells and necessary for the differentiation of these cells, and this reciprocal interaction can validate the associated increase of *TGFB* and *FOXP3* in this study. According to reports, the increased level of *IL-23*/IL-17 pathway is an important axis of inflammation and increase of IL-17 should be considered as an initiation factor for the disease [55]. We found that this cytokine increased significantly in the PBMCs of endometriosis patients, but it is not in line with Andreoli's study, probably because they selected patients with the early-stage of the disease [56].

With respect to PFMCs, we found that the cytokines expression had a similar pattern with PBMCs. This similarity may accentuate endometriosis has a systemic response associated with concentration in peritoneum. However, the increase in the expression level of *IL-17* and *TGFB* was significant. These findings are in agreement with those of Zhang et al. and Kyama et al. [54,57]. According to research, these two cytokines have crucial roles in some diseases with infertility consequences [58].

On the other hand, there were significant increases in IL-4, *TNFA* and *IFNG* mRNA expression in eutopic endometrium of patients in comparison with the control group. Antsiferova et al. indicated diminished mRNA expression of *IL-4* in eutopic endometrium of endometriosis patients. This is in disagreement with our results, although they did not mention staging of the patients [49]. Kyama et al. also found an increase in TNF-α [54], but there is a conflicting study that reported decrease of TNF-α level in endometriosis patients compared with healthy controls [59]. Our results concerning IFN-γ in eutopic endometrium provide more evidence for the other studies [60]. Increased TNF-α plays role in infertility and IFN-γ does likewise.

Comparing ectopic endometrium of patients with the endometrium of non-endometriosis women, we found that *IL-4* increased significantly, while *IL-23* and *IFNG* decreased dramatically. OuYang et al. also reported abundance of *IL-4* positive cells in stroma of ectopic endometrium in endometriosis patients [61]. Other researchers also indicated that *IL-4* and TGF-β1 increased in endometriotic lesions [49,62,63]. So, decreased level of inflammatory cytokines in extra-uterine lesions might indicate an overwhelming anti-inflammatory condition that facilitates establishment and expansion of the lesions. In agreement with our study, Mier-Cabrera et al. reported that increase of IFN-γ in mouse models could reduce weight and area of ectopic lesions [64]. Also, Szyllo et al. showed diminished secretion of IFN-γ from stimulated lymphocytes of endometriosis patients in comparison with control group. No article was found about expression of *IL-23* in ectopic lesions of endometriosis.

On the other hand, gene expression of *IFNG* significantly decreased in ectopic endometrium of endometriosis patients in comparison to eutopic endometrium of the same group. However, the result is in disagreement with that of Chiang et al., in that, they found enhanced IFN-γ expressing T cells in ectopic lesions compared to eutopic endometrium [65]. But Bergqvist's et al. finding was in accordance with our study [59]. This reduction, as well as decrease of *IL-23* and *TNFA*, may be the result of increased level of TGF-β, as this cytokine inhibits inflammation through suppressing Th1 and Th17 [63,66].

In summary, this work's findings along with the previous studies suggest that at first endometrial cells enter peritoneal cavity during retrograde menstruation. As displaced antigens, endometrial cells stimulate native immunity to send signals by producing cytokines. Then, by activating Th17 cells, neutrophilic inflammation initiates, which is intensified by Th1 cells. At this stage, inflammatory cytokines hurt the surrounding tissues. As a result of cyclic hormonal changes that lead to *GATA3* expression, activated Th2 cells produce anti-inflammatory cytokines to decelerate the inflammation. At last, gene expression of *FOXP3* as well as production of TGF-β activates Treg cells to subside inflammation and repair damaged tissues through fibrosis. After several years of repetitive increase and decrease of inflammation in every menstrual cycle, fibrosis and organ attachment take place. Indeed, no specific T cell subset alone could be responsible for the establishment and progress of endometriosis, but periodically all the four subsets engage in the process of endometriosis in turn and in a network manner. Disruption of this network mechanism could contribute to the progress of the disease stages in patients.

Conclusion

Comparison between endometriosis and non-endometriosis women shows Th17, Th1, Th2 and Treg cells through *IL-17*, TNF-α, *IL-4*, and TGF-β have influencing effect on the disease progress. While inflammatory T helper cells and cytokines are actively impressive in initiation and progression of endometriosis, suppressing T helper cells and cytokines may inhibit inflammation especially in endometriotic

tissues.

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