# T Helper Cells Profile and CD4$^+$CD25$^+$Foxp3$^+$Regulatory T Cells in Polycystic Ovary Syndrome

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## ABSTRACT

**Background:** Polycystic ovary syndrome (PCOS) is considered as the most common cause of female infertility that affects 4-10% of women in the reproductive age. Previous studies have shown the role of a balanced immune response in a successful pregnancy and fertility. **Objective:** To investigate the T helper cells type 1 (Th1) /Th2/Th17/Treg paradigms in peripheral blood of infertile PCOS compared with normal fertile women. **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated at the late follicular phase from 10 PCOS and 10 fertile women. PBMCs were stimulated with PMA and ionomycin in the presence of Berefeldin A as Golgi stop agent to detect intracellular cytokine production (IFN-γ, IL-17, and IL-4) from CD3$^+$CD4$^+$T cells population indicating T helper (Th) cells subsets by flowcytometry. Moreover, regulatory T cells were enumerated using CD25 and Foxp3 markers. **Results:** In this study, we report that the frequency of Th1 cells was increased compared to Th2 cells in infertile PCOS when considering Th1/Th2 ratio (P=0.05). Analysis of Th17/Th2 ratio showed a significant difference with a bias toward Th17 dominancy in PCOS (P=0.02). The proportion of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells was significantly lower in PCOS patients than that of healthy fertile women (P=0.02). **Conclusion:** In summary, Th1 and Th17 bias and reduction of Treg and Th2 cells as regulators of immune responses might be involved in the pathogenesis of PCOS. These results are suggestive of an altered immune response to inflammatory status in PCOS patients, likely causing some complications such as infertility in these patients.

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## INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 4-10% of women in the reproductive age (1,2). From a pathophysiology perspective, PCOS is a heterogeneous disorder characterized by ovulatory dysfunction, hyperandrogenism, and polycystic ovarian morphology (3). Moreover, PCOS is considered as one of the important causes of infertility worldwide. The neuroendocrine features of this disorder include increased serum concentration of luteinizing hormone (LH), decreased level of progesterone, and increased estrogen secretion (4,5). Sex steroidal hormones are implicated in the immune responses and might modulate both pro- and anti-inflammatory immune responses coordinated by T helper subsets (6). Immune dysregulation due to hormonal imbalance may play an important role in the pathogenesis of PCOS. Previous studies have shown that hormonal imbalances might trigger the production of a group of autoantibodies including, anti-nuclear, anti-thyroid, and anti-islet cell antibodies in PCOS women (7). Moreover, increased number of white blood cells has been reported in PCOS along with low-grade inflammation. In this regard, endothelial dysfunction increased oxidative stress, and elevated levels of C-reactive protein, IL-6, IL-18, monocyte chemoattractant protein-1 have been also reported (8,9). Interestingly, the immunological bases of many pregnancy disorders and infertility show great similarities. Same as several pregnancy complications such as recurrent abortion and pre-eclampsia increased T helper type 1 (Th1) and decreased Th2 responses have been reported in PCOS (10,11). In the recent years, the importance of distinct Th cell subsets in the regulation of immune response, especially in the context of pregnancy has been demonstrated. Indeed, it seems that the fine balances in Th1/Th2/Th17/Treg activities will define the fate of the pregnancy (12). There are few publications on the measurement of cytokines related to Th responses in PCOS women. To the best of our knowledge, there is no published data regarding the investigation of Th1/Th2/Th17/Treg balances within peripheral blood lymphocytes. In the present study, we aimed to investigate the distribution of Th1/Th2/Th17/Treg subsets in the peripheral blood of infertile women complicated with PCOS compared with healthy fertile women. To achieve this goal, we investigated the frequencies of CD4+ Th subsets using the flow cytometry technique.

## MATERIALS AND METHODS

**Subjects.** Ten women diagnosed with PCOS (according to Rottedam criteria) and 10 healthy fertile women were enrolled in the present study. The sample size was decided by consulting with a statistician and according to the sample sizes of the similar previously published papers (13). The study was performed at the division of reproductive immunology, department of immunology, Shiraz University of Medical Sciences. All participants signed an informed consent. Moreover, the study was approved by the local Ethics Committee of Shiraz University of Medical Sciences. None of the participants received any treatment including intrauterine insemination (IUI) or *in vitro* fertilization (IVF) and also medication at the time of sampling. Inclusion criteria for PCOS cases were clinical and/or biochemical signs of hyperandrogenism, oligo/ or anovulation polycystic morphology of the ovary, having no active infection, and no history for autoimmune diseases or malignancy. None of the subjects was

pregnant and none had a history of delivery. Healthy fertile women were selected from multiparous non-pregnant women with no history of any complicated pregnancy or abortion who had at least two previous successful deliveries. Exclusion criteria for this group were an active infection and history of autoimmune diseases and malignancy. Clinical and biochemical features of the PCOS patients and healthy fertile women are shown in Table 1 and Table 2, respectively.

**Table 1. Clinical features of the PCOS patients and normal fertile women.**

|  | Control (n=10) | PCOS (n=10) | *P-value*[a] |
|---|---|---|---|
| Age (year) | 27 ± 0.6 | 27 ± 1.2 | 0.6 |
| BMI (Kg/m$^2$) | 25.2 ± 0.9 | 25.8 ± 1.2 | 0.7 |
| Regular menstrual rate | Yes | No | <0.05 |
| Hirsutism score | 0 | 6.8 ± 3 | <0.05 |
| Duration of infertility (year) | NO | 4.3 ± 0.39 | <0.05 |
| Child of birth | 2 ± 1 | NO | <0.05 |

[a]Two-tailed *t-test* was applied for comparisons of differences between women with PCOS and normal fertile women. Data are means ± SEM. P-value <0.05 considered as statistically significant. PCOS: Polcystic ovary syndrome.

**PBMC Isolation and Cryopreservation.** Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml of heparinized venous blood using Ficoll-Hypaque (Axis-Shield, Oslo, Norway) density gradient centrifugation. After separation, PBMCs were counted and frozen in a cryoprotective media containing 10% dimethyl sulfoxide (DMSO) (Shellmax, China) and fetal bovine serum (FBS) (Shellmax, China) and stored in liquid nitrogen (LN2) until the time of the experiment. The cell storage period in LN2 was less than one month for all samples.

**Table 2. Hormone concentrations in PCOS and normal fertile women.**

|  | Control (n=10) | PCOS (n=10) | *P*-value [a] |
|---|---|---|---|
| TSH (mIU/ml) | 2.2 ± 0.2 | 2.05 ± 0.2 | 0.6 |
| FSH (mIU/ml) | 4.7 ± 0.04 | 6.6 ± 1 | 0.1 |
| LH (mIU/ml) | 5.8 ± 0.8 | 8.4 ± 1.7 | 0.05[*] |
| Prolactin (ng/ml) | 16.07 ± 1.9 | 23.26 ± 0.6 | 0.5 |
| Free testosterone (ng/dl) | 0.59 ± 0.05 | 1.03 ± 0.3 | 0.9 |
| DHEAs (µg/ml) | 1.02 ± 0.1 | 1.25 ± 0.1 | 0.3 |
| AMH (ng/ml) | 4.8 ± 0.9 | 7.1 ± 1 | 0.03[*] |
| FBS (mg/dl) | 90 ± 1.6 | 87 ± 1.4 | 0.3 |
| Fasting insulin (µU/ml) | 8.3 ± 1.1 | 13.8 ± 1.6 | 0.006[**] |

[a]Two tailed *t-test* was applied for all hormone analysis. Data are means ± SEM. P-value <0.05 considered as statistically significant. PCOS: polycystic ovary syndrome, DHEAs: dehydroepiandrosterone sulfate. AMH: Anti-mullerian hormone.

**Short-term Cultures and Stimulation.** To detect intracellular cytokines, short-term culture and stimulation were performed based on a method designed and optimized in our lab. Briefly, PBMCs were removed from LN2 and rapidly thawed and washed twice with RPMI 1640 (Shellmax, China) supplemented with 10% FBS (Shellmax, China)

and 1% penicillin-streptomycin (Shellmax, China). A total of $1\times10^6$ cells/well were cultivated in 24-well flat-bottom plates in 1 ml complete RPMI 1640 media. The cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich, USA) plus 250ng/ml Ionomycin (Sigma-Aldrich, USA), and incubated at 37℃ in a humidified 5% CO2 incubator for 16 hours. Moreover, Berefeldin A (0.0016 mg/ml or 0.25x) as Golgi stop agent (Biolegend, San Diego, CA, USA) was added 1 hour after stimulation of the cells with PMA and Ionomycin. In each experiment for every stimulated tube, an unstimulated condition was considered as the negative control.

**Flow cytometry.** Cells were harvested from each condition and were washed twice with PBS. A total of $7\times10^5$ cells were re-suspended in 100 µl cold PBS and were incubated with monoclonal antibodies directly conjugated with different fluorochromes including CD3-PerCP, CD4-APC, and CD25-FITC (all purchased from BD, Pharminogen, USA) for 20 minutes at 4°C in the dark for surface markers detection. For intracellular staining, human Foxp3 buffer set kit (BD, Pharminogen, USA) was used. Briefly, cells were supplemented with fixation solution for 15 min, followed by adding permeabilization solution and incubating it for 30 min at room temperature. FITC-conjugated anti-human IFN-γ, PE-conjugated anti-human IL-4, IL-17, and Foxp3 antibodies (all purchased from BD, Pharminogen, USA) were added and incubated for 30 min at room temperature in the dark. After staining, cells were washed and re-suspended into 1% formaldehyde fixation solution (Merck, USA).

**Acquisition and Analysis of Flowcytometric Data.** The stained and fixed samples were analyzed using a 4 color FACSCalibur flow cytometer and data were analyzed using Cell Quest software (BD Biosciences, San Joes, California, USA). The number of events acquired for each sample was 100,000 events. Live lymphocytes were gated based on the Forward and Side scatter. Then, lymphocyte region was used to obtain the gated fluorescence plot for CD3 versus CD4 in all unstimulated and stimulated samples. All data were expressed as the percentage of cytokine-positive CD3+CD4+ cells, and CD3+CD4+CD25+FoxP3+ cells were considered as conventional regulatory T cells.

**Statistical Analysis.** The statistical analysis was performed using GraphPad Prism 5 software (La Jolla, California, USA) and SPSS 18 software. One-Sample Kolmogorov-Smirnov and Levene's test were used to examine the normal distribution of the variables and homogeneity of variances, respectively. An unpaired two-tailed t-test was applied for comparing the percentages of CD4+ T cell subsets between groups. The ratios of Th cell subsets were also determined and compared between the two studied groups by using unpaired two-tailed t-test. Spearman's rank correlation coefficient was used to analyze the relationship among the proportion of Th1, Th17, and Treg/Th2 subsets.

## RESULTS

**The Frequencies of CD3+CD4+T Cells.**
First, the percentages of peripheral blood CD4+ T lymphocytes were determined by flow cytometry analysis. Results indicated no statistically significant differences in percentages (%) of CD3+CD4+ cells between infertile women complicated with PCOS and healthy fertile women (Mean% ± SEM: 28.2% ± 4.1 and 27% ± 2.5 respectively, P=0.83, Figure 1). The similarity in the percentages of CD3+CD4+ T cells between PCOS and healthy fertile women indicated that total population of CD4+ T cells in both studied groups were the same. Because of endocytosis of CD3 and CD4 molecules after
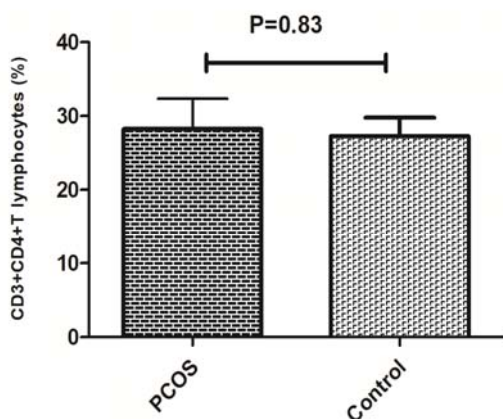
**Figure1. Percentages (%) of CD3+ CD4+T cells between women with PCOS as compared with healthy fertile women.** Data are expressed mean% ± SEM. Two-tailed *t*-test was applied for comparisons of differences in percentage of CD3+ CD4+ T cells between women with PCOS and normal fertile women. P-value <0.05 considered as statistically significant. PCOS: Poly cystic ovary syndrome.

PMA and ionomycin stimulation, gating strategy for flow cytometric analysis was set on CD3+CD4+ T cells in unstimulated condition. The latter gating strategy in stimulated condition was considered to ensure the accuracy of the cytokine-secreted CD3+CD4+ cell population. The geometric mean fluorescent intensity for CD4 and CD3 molecules in stimulated and unstimulated condition are presented in Figure 2.
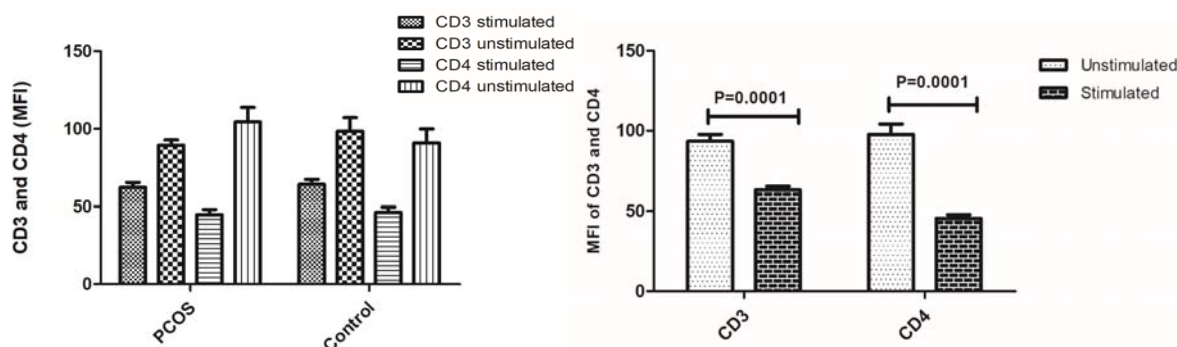


**Figure 2**. **The Geometric mean florescent intensity (MFI) of CD4 and CD3 molecules**. Data was shown in the conditions that were done- in the absence of PMA and ionomycin substances (Unstimulated condition) and in the presence of PMA and ionomycin (Stimulated condition) to stimulate PBMCs derived from both PCOS and normal fertile women. Data are expressed as mean ± SEM. Two-tailed *t*-test was applied for comparisons of differences between MFI of CD3 and CD4 molecules expression in stimulated and unstimulated conditions. P-value <0.05 was considered as statistically significant.

**The Proportion (%) of Th1, Th2, Th17, and TregSubsets.**
The gating strategy for evaluation of Th1, Th2, Th17, and Treg subsets is shown in Figure 3. IFN-ɣ, IL-4, and IL-17 positive cells, which indicate Th1, Th2, and Th17 cell subpopulations, respectively, were measured in unstimulated and stimulated conditions. Results showed that about 2-3% of CD3+CD4+ cells expressed these cytokines before stimulation in both groups. After stimulation, the proportion of both Th1 and Th2 cells were significantly decreased in women with PCOS as compared with normal fertile women (P=0.01 for Th1, and P=0.004 for Th2).
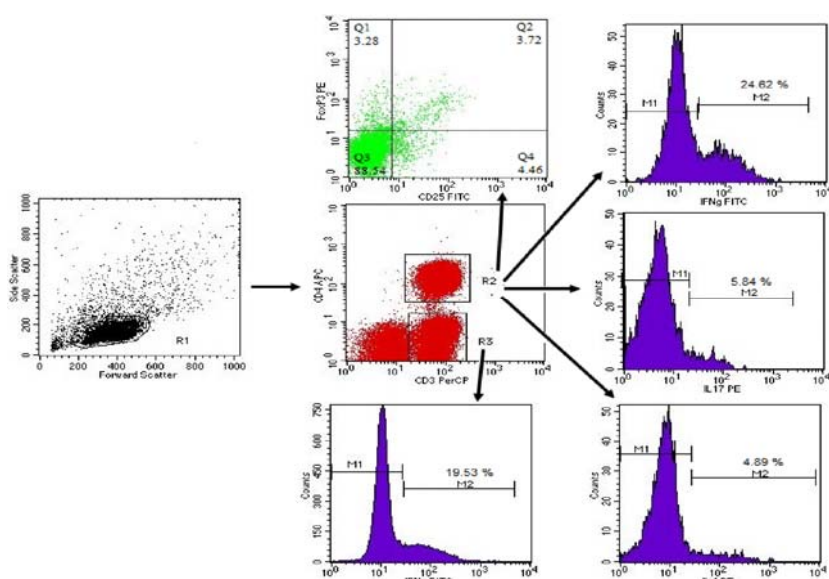
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**Figure 3. Four color flow cytometric analysis of Th1/Th2/Th17/Treg in the peripheral blood.** For the gating strategy, lymphocytes (R1) were gated based on the forward and side scatter parameters, then CD3+ CD4+ (R2) or CD3+CD8+ T cells (R3) were selected of the lymphocyte gate. Then, intracellular cytokine expression of IFN-γ, IL-17 and IL-4 was evaluated from CD3+CD4+ populations. Moreover, CD25 and Foxp3 markers were plotted against each other and the frequency of CD25+Foxp3+ regulatory cells was determined in the CD3+CD4+ T cells. We also determined intracellular expression of IFN-γ from CD3+CD8+ populations.

Although the percentage of Th17 cells in PCOS patients was increased compared to healthy fertile the women, this difference was not statistically significant (P=0.5). We considered CD3+CD4- region as CD3+CD8+ cells, then the production of IFN-γ was analyzed in this population. Results indicated that PCOS women produce more IFN-γ compared with healthy ones; however, the difference was not statistically significant (P=0.6). The proportion (%) of CD3+CD4+CD25+Foxp3+ regulatory T cells was significantly lower in women with PCOS compared with that of normal fertile women (P=0.02). The data for this part are presented in Table 3.

**Table 3. The proportion (%) of CD3+CD4+ (T helper) lymphocytes and CD3+CD4-(CD8+ T cells) in women with PCOS and normal fertile groups.**

| Th Subsets | PCOS (n=10) | Control (n=10) | *P*-value[a] |
|---|---|---|---|
| Th1(IFN-γ) | 18 ± 2.8 | 31.66 ± 3.7 | 0.01 |
| Th2 (IL-4) | 7.9 ± 1.4 | 16.7 ± 2.2 | 0.004 |
| Th17(IL-17) | 7.9 ± 1.7 | 6.5 ± 0.9 | 0.5 |
| Treg(CD4+CD25+Foxp3+) | 3.1 ± 0.5 | 6.8 ± 1.4 | 0.02 |
| TCD8+(IFN-γ) | 19.49 ± 2.4 | 17.75 ± 2.9 | 0.6 |

[a]Comparisons were made between women with PCOS and normal fertile controls using two-tailed *t*-test. Data are means ± SEM. P-value <0.05 was considered as statistically significant.

## T helper Cells Subset Ratios.

Th1/Th2 and Th17/Th2 ratios were compared between PCOS patients and healthy fertile women. We observed a significant difference in the Th1/Th2 ratio (P=0.05) and the Th17/Th2 ratio (P=0.02), with a bias toward Th1 and Th17 in PCOS patients. As shown in Figure 4, the analysis of Th1/Treg and Th17/Treg ratios showed no significant difference between PCOS patients and healthy fertile women (P=0.7 and P=0.1, respectively).
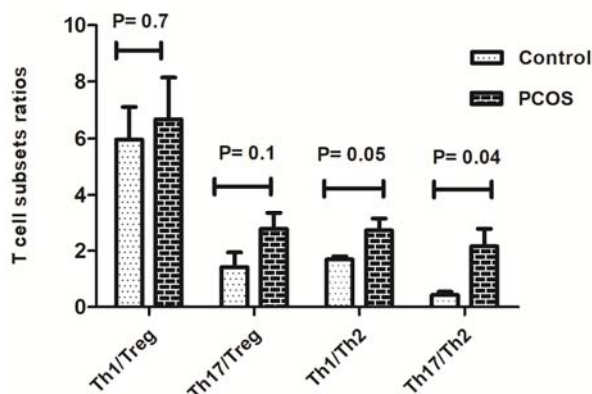


**Figure 4. Comparison of T helper 1 (Th1)/Treg, Th17/Treg, Th1/Th2, and Th17/Th2 in women with PCOS (n=10) and normal fertile women (n=10).** Values are mean% ± SEM. Two-tailed *t*-test was applied for comparisons of differences between women with PCOS and normal fertile women. P-value <0.05 was considered as statistically significant. Treg: regulatory T cells, P: P-value.

## T helper Cell Subsets Combination Ratios.

We pooled the percentages of inflammatory (Th1 and Th17) and anti-inflammatory or regulatory cells (Th2 and Treg) and compared the results between PCOS and healthy women. Results revealed that, in general, the ratio of inflammatory to anti-inflammatory or regulatory subsets was increased in women with PCOS complication compared to healthy women. In combination analysis, although the results indicated a bias toward inflammatory responses in PCOS women, none of the comparisons indicated significant differences (Figure 5).
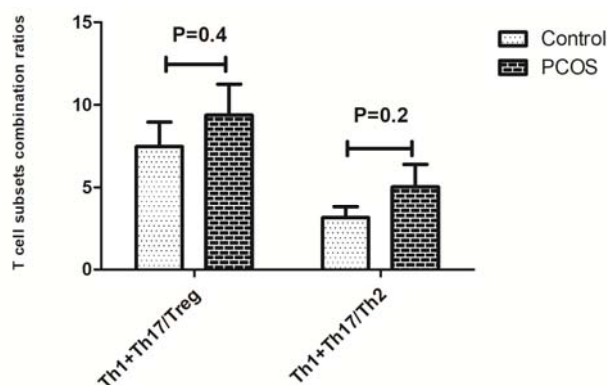


**Figure 5. Comparison of T helper 1 (Th1)+ Th17/Treg, Th1+Th17/Th2 in women with PCOS (n=10) and normal fertile women (n=10).** Values are mean% ± SEM. Two-tailed *t*-test was applied for comparison of differences between women with PCOS and normal fertile women. P-value <0.05 was considered as statistically significant. Treg: regulatory T cells, P: P-value.

**Correlation between T helper Subsets.**
In women complicated with PCOS, negative correlations were detected between Th1 and Treg, Th1 and Th2, and also Th17 and Th2 subsets. In addition, a positive correlation was detected between Th17 and Treg in women complicated with PCOS. Interestingly, in healthy women compared to PCOS patients, positive correlations were observed between Th1 and Treg, and also Th1 and Th2. Moreover, negative correlations were observed between Th17 and Treg and Th17 and Th2. Although not all correlations were statistically significant, they showed an inflammatory condition in PCOS patients.

## DISCUSSION

In the present study, we investigated the frequencies of three T helper subsets including Th1/Th2/Th17 and also the frequency of Treg cells during the late follicular phase (days 8-13) in the peripheral blood of patients with PCOS compared with healthy controls. The follicular phase was selected because, at this phase of menstrual cycles, FSH and estradiol secretion are at the highest levels (15). We used flow cytometry to evaluate these subsets. In general, the results of the present study indicated a bias toward inflammatory and away from T regulatory cells in PCOS women. We reported that there is a significant bias toward Th1 responses in women complicated with PCOS when considering Th1/Th2 ratio. In line with our data, previous studies showed the importance of Th1/Th2 ratio in other pregnancy disorders. For example, Kim *et al.* reported that the ratio of Th1/Th2 immune responses is more important than the expression of a single cytokine in infertile women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF (10). Considering a single T helper subset (a single cytokine), we reported a significant decrease in Th1 cytokine in PCOS patients compared with healthy fertile women. On the contrary, Qin *et al.* reported increased Th1 cytokines in the follicular fluids from PCOS women (14). Moreover, they also reported a significant decrease in Th2 cytokines that is consistent with the results of the present study (14). In this regard, our results indicated the importance of Th1/Th2 balance in PCOS women. Considering the inflammatory basis of PCOS, we also evaluated Th17 subset. Previous studies indicated that inflammatory cytokines such as IL-6 and IL-18 were increased in PCOS women (8,9). Furthermore, an interesting study reported a relation between IL-17 and PCOS (16). In line with the mentioned studies, we observed an increase in the Th17 subset in PCOS women, although the difference was not statistically significant. However, analysis of Th17/Th2 ratio showed a significant difference with a bias toward Th17 dominancy. Recent data show that Th17 cells participate in pregnancy-related pathologies, including recurrent spontaneous abortion (RSA)(17-19) and pre-eclampsia (PE) (20,21). Moreover, we observed a negative correlation between Th17 and Th2 subsets in PCOS patients. Our data indicated that Th17 cells might emerge as an important mediator of inflammation and tissue damage in PCOS.
Finally, we investigated the frequency of CD4+ regulatory T cells. Regulatory T cells are the most important subset of CD4+ T cells essential to maintain maternal-fetal tolerance during pregnancy to facilitate a successful implantation (22,23). Decreased percentage of CD4+ regulatory T cells are reported in several pregnancy-related diseases such as spontaneous recurrent abortion (24) and preeclampsia (25). The results of the present study demonstrated a significant reduction in the percentage of

CD4+CD25+Foxp3+ T cells in PCOS compared with healthy women. Our observation of diminished Treg frequency in PBMCs strongly supports a previous report published in 2015 (26). They showed that the numbers and expansion of CD4+FoxP3+CD25+CD127-/low cells were reduced in PBMCs of PCOS patients in the follicular phase (26). Furthermore, we found a negative correlation between Th1 and Treg subsets in PCOS women. Although this negative correlation was not statistically significant in patients, it showed an inverse trend compared to healthy women. So, our results showed a disturbance in Th1/Treg balance in women complicated with PCOS. Moreover, we investigated Th1/Treg and Th17/Treg balances in PCOS patients compared with normal fertile women. Our data showed that Th17/Treg balance is more important in the context of PCOS. However, further studies with a larger sample size will shed light on this matter. A previous study in this regard demonstrated an inverse relationship between Th17 cells and regulatory T cells and deregulation of Th17 cells by regulatory T cells in early pregnancy in unexplained recurrent miscarriage patients (27).

In conclusion, the study of effector Th cells is one of the most fascinating areas in immunology. Regarding Th17 cell involvement in most diseases, the former Th1/Th2 paradigm of pregnancy was modified into Th1/Th2/Th17/Treg paradigms, each of which involves complicated interactions with decidual NK cells, dendritic cells, monocytes, and extravillous trophoblast cells (28). In this study, we propose that apart from Th1/Th2 and Treg cells, Th17 cells play a role in the immune-pathogenesis PCOS. In the present study, Th1/Th2 and Th17/Treg imbalances were observed in PCOS patients and found the notion that women with normal pregnancy (multiparous women) have a Th2 bias along with normal Treg cell frequency, while infertile women with PCOS have a bias toward Th1-type reactivity with a decrease in Treg cells. Finally, it is of note the present study is specific to women with PCOS who had no history of IUI or IVF stimulation. PBMCs stimulation was performed with PMA and ionomycin. The results demonstrated a pre-existing Th1 and Th17 shift that may be induced by stress and high levels of female sex hormones. Therefore, further studies are needed to explore the relationship between T helper cell cytokine regulations in the presence of trophoblast antigen stimulation. Moreover, to obtain more valuable findings, it is necessary to consider a larger number of Th-related cytokines subsets (especially TNF-α, and IL-10).

## ACKNOWLEDGEMENTS

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