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Comparing the Frequency of CD4+T Cells in Recurrent Spontaneous Abortion Women with and without Anti-thyroid Peroxidase (TPO)

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ABSTRACT

Thyroid autoimmunity, being recognized by the presence of auto-antibodies against thyroid peroxidase (TPO) and thyroglobulin, has known to be associated with increased risk of recurrent spontaneous abortion (RSA), even in euthyroid subjects. There was no robust evidence regarding T cell deviations in anti-TPO positive RSA patients. The aim of this study was to investigate if the numbers of different CD4+T subsets were different in women who experienced RSA and have an anti-TPO antibody from those without autoantibody and normal fertile women or not.

In this study, peripheral blood samples were obtained from three groups of women (age: 20-35 years) including RSA anti-TPO positive (n=17), RSA anti-TPO negative (n=27), and fertile (n=29) groups. The frequency of T helper (Th) 1, Th2, Th17, and regulatory T cells (Tregs) and also, the proportions of Th1/Th2 and Th17/Treg were measured by flow cytometry and compared between groups in different menstrual phases.

The findings indicated elevated levels of Th1 in anti-TPO+ RSA in comparison with those without anti-TPO (p-value: 0.004), exclusively in the luteal phase. Other T cell subsets were different only between RSA and control groups. Also, the Th1/Th2 and Th17/Treg ratios were increased in both RSA groups compared to fertile women.

The only subset of CD4+ T cell different between RSA groups (i.e. with and without anti-TPO) was Th1 cells. Other CD4+ T cells' deviations including Th2, Th17, and Treg cells could be related to the presence of abortion, regardless of the underlying thyroid autoimmunity state.

Keywords: Helper T cells; Regulatory T cells; Recurrent abortion; Thyroid peroxidase activity-inhibiting immunoglobulins

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INTRODUCTION

Recurrent spontaneous abortion (RSA) is a challenging problem, defined as two or more pregnancy loss before the 20th week of gestational age.[1] Despite considerable developments in the diagnosis of RSA causes, approximately 50% of its etiology is unknown.[2]

Thyroid autoimmunity; being associated with the pathogenesis of RSA, is recognized by the presence of auto-antibodies against thyroid antigens involving thyroid peroxidase (TPO) and thyroglobulin.[3] In some occasions, this autoimmunity leads to thyroid dysfunction; however, even in euthyroid subjects, the presence of these autoantibodies could be a risk factor for pregnancy morbidities such as RSA and preterm birth.[4]

One of the proposed mechanisms for this contribution; especially in women with normal thyroid function, is the likelihood of deviations in cell-mediated immunity.[5] Previous studies have shown that cell-mediated immune disturbances are attributed to RSA pathogenesis. Deviation of T helper (Th)1, Th2, Th17, and regulatory T (Treg) cell numbers has been considered as a cause of recurrent pregnancy loss [6-11] however, it is not clear whether this deviation is also seen in euthyroid women who had anti-TPO and the history of RSA or not.

The aim of this study was to investigate if the numbers of different CD4+T subsets were different in women who experienced repeated miscarriage and have anti-TPO from those without autoantibody and normal fertile women or not.

PATIENTS AND METHODS

All of the sampling methods and experiments used in this study were approved by the Ethics Committee of Tehran University of Medical Sciences (Ethical code: IR.TUMS.MEDICINE.REC.1395.2622). Written informed consent was obtained from each participant prior to sampling.

Participants' Characteristics

In this study, peripheral blood samples were obtained from three groups of women referred to Yas Women Hospital affiliated to Tehran University of Medical Science (September 2016-November 2017). These groups consisted of RSA women with anti-TPO, RSA women without anti-TPO and Fertile control women (without determination of anti-TPO levels).

All heparinized venous blood samples were taken from non-pregnant women aged 20-35 years old. All the participants, including RSA and fertile women, were euthyroid (TSH<5mIU/L, normal free T4). In RSA women, patients experienced at least two consecutive pregnancy losses before the 20th week of gestation. According to anti-TPO levels, these women were categorized to anti-TPO positive group (anti-TPO≥35 IU/mL, anti-TPO+); including 17 samples (age: 29.06±4.72 years), and anti-TPO negative group (Anti TPO<35, Anti-TPO-); consisted of 27samples (age: 29.89±3.23 years). Group 3 (Fertile control women) consisted of 29 healthy euthyroid volunteers (age: 30.17±3.39) with at least one live birth and no history of abortion or stillbirth in previous pregnancies. None of the women in any of the groups had a detectable active autoimmune disease.

Patients with a history of pregnancy loss due to anatomical, endocrine, and genetic causes or infectious agents were excluded from the study. Thyroid function tests were checked by ELISA. The menstrual phase of blood sampling was determined based on the reported last menstrual period.

Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated; using Ficoll-Hypaque (Inno-train, Germany) density gradient centrifugation. Separated cells were stained with Trypan blue dye and the cell counts and viability were examined microscopically. The cells were frozen in freezing media contained 10% Dimethyl sulfoxide (DMSO) (Roth, Germany) in fetal bovine serum (FBS)(Gibco, UK) and stored at -70°C.

After collecting all samples, PBMCs were thawed, cleared of DMSO completely, and examined for their viability by Trypan blue staining. Then, appropriate viable PBMCs were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Biosera, France) supplemented with 10% FBS (Gibco, UK), 100U/mL penicillin, and 100 µg/mL streptomycin (Gibco, UK) in 24 well cell culture plates. Cells were seeded into each well at the density of 1.5×10^6 cells/mL and were incubated at 37°C, 5% CO2 incubator for six hours. To activate PBMCs, 50 ng/mL phorbol myristate acetate (PMA)(Sigma, USA) and 1µg/mL ionomycin (Sigma-Aldrich, USA) was added to the wells at the beginning of the culture incubation. One hour later, 10µg/mL Brefeldin A (eBioscience, USA) was added to inhibit cytokine secretion.

Flowcytometry Analysis

After cells were harvested from cell culture plates, extracellular as well as intracellular staining was performed with anti-human fluorophore-conjugated mouse monoclonal antibodies (Biolegend, USA) to determine Th1, Th2, Th17, and Treg cells. For analyzing Treg cell frequencies, 0.5×10^6 unstimulated PBMCs were first stained with anti-CD4 FITC-, anti-CD25 PE/Cy7-, and anti-CD127 APC-conjugated antibodies. After cell fixation and permeabilization, cells were stained with anti-FOXP3 PE-conjugated antibody. For the investigation of other T helper subsets (Th), stimulated cells were stained with anti-CD4 FITC-conjugated antibody, followed by fixation, permeabilization and intracellular staining with anti-IFN-γ PE/Cy7-, anti-IL-4 APC, and anti-IL-17A PE-conjugated antibodies. Isotype-matched fluorophore-conjugated monoclonal antibodies were used as controls. Then, 10^5 cells were counted and examined with a BD FACSCalibur instrument (Beckton Dickinson, USA). Collected data were analyzed with FlowJo software (version 7.6, UK).

Gating Method

We used two methods for gating of stimulated and unstimulated cells for detecting different subsets of CD4+T cells. First of all, the lymphocytes were gated based on forward and side scatter. Then amongst the stimulated cells, CD4+IFN-γ+, CD4+IL-4+, and CD4+IL-17+ cells were considered as Th1, Th2, and Th17 cells, respectively (Figure 1).

For determining the frequency of Treg cells, we used unstimulated cells and CD4+CD127- cells were selected from gated lymphocytes. Then, from these CD4+CD127- cells, CD25+FOXP3+ were specified. The frequencies of these cells (CD4+CD25+FOXP3+CD127-) amongst lymphocytes were calculated according to statistics in flowJo software (Figure 2).

Although it was better to use the anti-CD3 antibody for specifying T cells, we did not use it. As the majority (not all) of stimulated CD4+IFN-γ+, CD4+IL-4+, and CD4+IL-17+ in gated lymphocytes are Th1, Th2, and Th17 cells, respectively, we considered them as these populations. A similar trend was applied for specifying CD4+CD25+FOXP3+CD127- as Treg cells.

Statistical Analysis

To compare the frequencies of Th1, Th2, Th17, and Tregs amongst three groups, we used Kruskal-Wallis and Mann-Whitney. The *p*-values ≤ 0.05 were considered statistically significant. We used SPSS (version 16, USA) and GraphPad Prism (USA) software for data analyzing and drawing the plots.

RESULTS

In this study, three different groups were studied which included anti-TPO^+RSA, anti-TPO^-RSA, and control groups. In each group, we had samples of both follicular and luteal phases.

The endocrinological and gynecological characteristics of participants were shown in table 1.

Comparing the Frequency of Th1 Cells between Three Groups

CD4+IFN-γ+ cells were considered as Th1 cells (Figure1). Comparing flow cytometry results showed that the proportion of Th1 cells was increased in anti-TPO^+RSA group compared to both anti-TPO^-RSA and fertile control groups exclusively in the luteal phase (Table 2, Figure 3). The Th1 cells' frequencies were not different between groups in the follicular phase.

Table 1. Endocrinological and gynecological characteristics of three groups' participants

	Anti TPO§+RSA§§ n=17 (F*=4, L**=13)	Anti-TPO- RSA n=27 (F=15, L=12)	Control n=29 (F=13, L=16)	*p*-value
Number of abortions(Median)	2	3	0	<0.001
	Min: 2, Max: 6	Min: 2, Max: 4		
Age (years)(Mean±SD^)	29.06±4.72	29.89±3.23	30.17±3.39	0.609
TSH***(uIU/mL)(Mean±SD)	2.17±1.01	1.91±0.99	1.98±0.95	0.512
Anti-TPO(IU/mL)(Mean±SD)	71.71±33.4	19.34±6.81	not measured	0.004

§ Thyroid peroxidase, §§ Recurrent Spontaneous Abortion, *F: Follicular phase, **L: Luteal phase, ^: Standard Deviation,

*** Thyroid Stimulating Hormone

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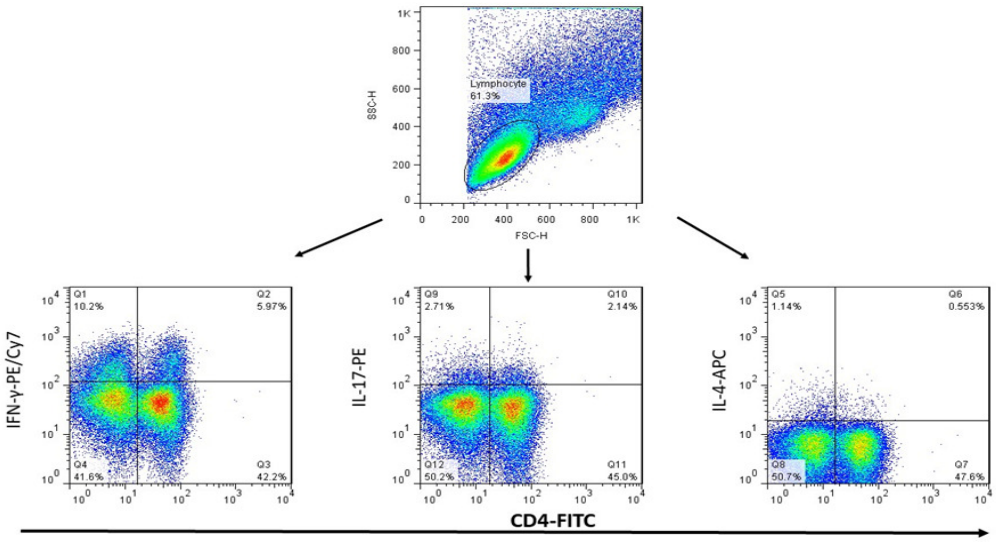

Figure 1. Gating strategy for detecting T helper (Th)1, Th2 and Th17 cells. Lymphocytes were gated based on forward and side scatter parameters (upper); CD4+IFNγ+ cells (lower left), CD4+IL-17+ (lower middle) and CD4+IL-4+ (lower right) were considered as Th1, Th17 and Th2 cells, respectively.

Comparing Frequency of Th2 Cells between Three Groups

CD4+IL-4+cells were considered as Th2 cells (Figure 1). Our findings indicated that Th2 cells were decreased in both RSA groups compared to the control group in luteal as well as the follicular phase. However, the frequency of these cells was not different between anti-TPO+ and anti-TPO- RSA groups in either phase (Table 2, Figure 3).

Comparing Frequency of Th17 Cells between Three Groups

CD4+ IL-17+ cells were considered as Th17 cells (Figure 1). Th17 cells were increased in anti-TPO$^-$ RSA compared to the control group in the follicular phase. Also, Th17 cells were upraised in both anti-TPO$^+$ and anti-TPO$^-$ RSA groups in comparison with the control group in the luteal phase. However, Th17 cell numbers were not different between anti-TPO positive and negative RSA groups in either phase (Table 2, Figure 4).

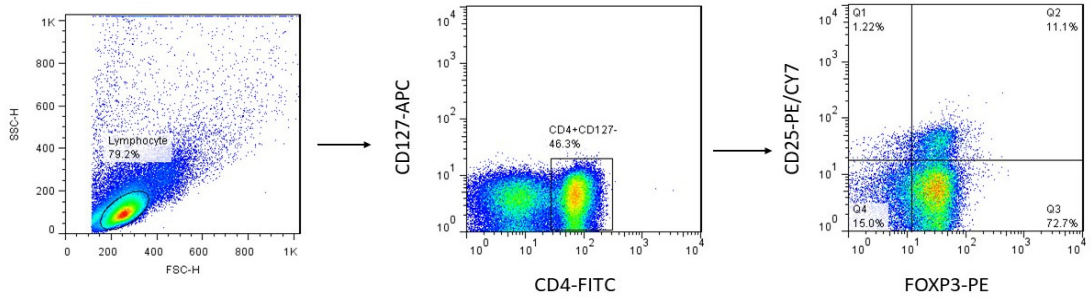

Figure 2. Gating strategy for detecting Regulatory T (Treg) cells. Lymphocytes were gated based on forward and side scatter parameters (left); from these lymphocytes, CD4+CD127- cells were gated (middle). Amongst CD4+CD127-cells, the CD25+FoxP3+cells (right) were determined as Treg cells and their frequencies were calculated from the original lymphocyte gate.

Table 2. Frequency of CD4+ T cell subsets in different study groups in different phases of the menstrual cycle

CD4+ T cells	Phases	Anti-TPO$^{\S+}$RSA§§	Anti-TPO^{-}RSA	Control	*p*-value
Th*1	Follicular	11.47 (8.74- 13.47)	9.07 (5.61-12.6)	8.27 (5.03-10.2)	0.353^{a} 0.054^{b} 0.530^{c}
	Luteal	11.72 (7.44-14.3)	7.31 (5.89-12.1)	7.73 (5.07-12.3)	1.00^{a} **0.003**^{b} **0.004**^{c}
Th2	Follicular	1.95 (1.82-2.22)	2.02 (1.34-2.8)	3.69 (2.31-4.14)	**<0.001**^{a} **0.012**^{b} 1.00^{c}
	Luteal	2.03 (1.45-2.69)	1.79 (1.39-2.7)	3.41 (2.38-4.5)	**<0.001**^{a} **<0.001**^{b} 1.00^{c}
Th17	Follicular	3.40 (2.53-4)	3.44 (2.92-4.88)	2.30 (1.93-2.73)	**<0.001**^{a} 0.054^{b} 1.00^{c}
	Luteal	3.86 (2.81-4.76)	3.32 (2.97-3.93)	2.33 (1.65-3.93)	**0.004**^{a} **<0.001**^{b} 0.57^{c}
Treg**	Follicular	3.33 (2.47-3.85)	3.42 (1.99-5.8)	5.1 (2.44-6.96)	**0.007**^{a} 0.062^{b} 1.00^{c}
	Luteal	3.54 (2.11-4.41)	3.33 (1.43-5.38)	5.50 (2.58-6.72)	**0.001**^{a} **<0.001**^{b} 1.00^{c}

Data were represented as percent of lymphocytes, Median (25 percentile-75 percentile), §: Thyroid peroxidase, §§: Recurrent Spontaneous Abortion, *: T helper, **: Regulatory T Cell, a. Anti-TPO- against control, b. Anti-TPO^{+} against control, c. Anti-TPO^{+} against Anti-TPO^{-}

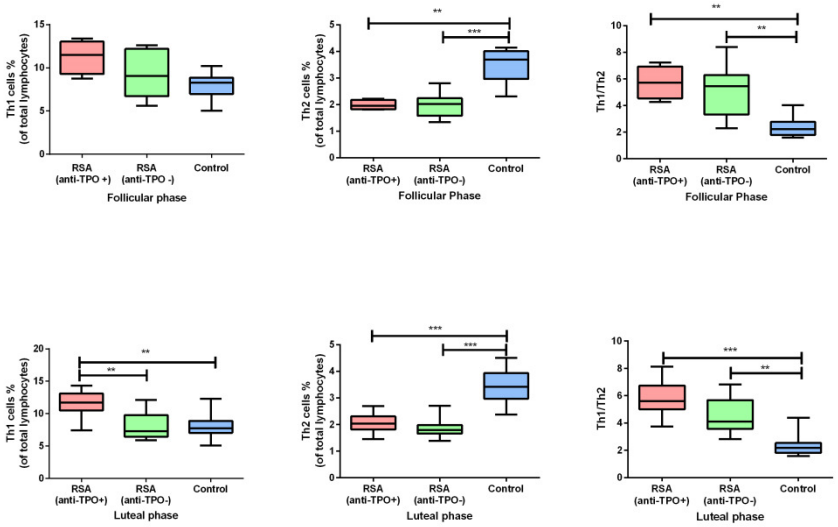

Figure 3. Percentile of T helper (Th) 1, Th2, and the ratio of Th1/Th2 in Follicular (upper row) and Luteal (lower row) phases in different study groups. Each box plot represents 25%-75% quartiles with the median. **: 0.001≤*p*-value≤0.05), *: *p* value<0.001**

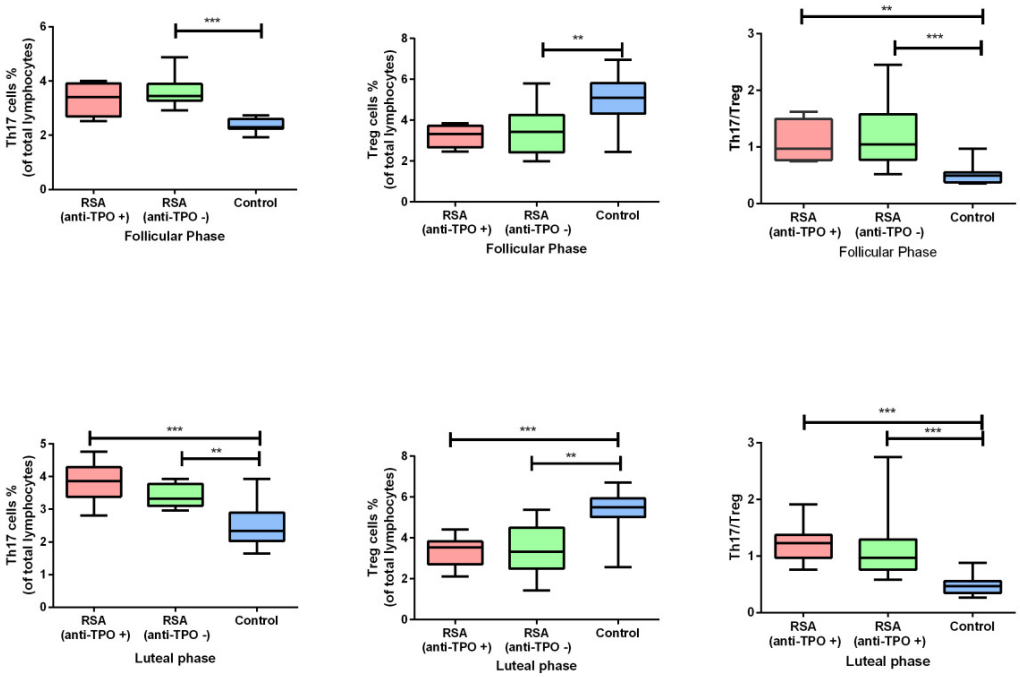

Figure 4. Percentile of T helper (Th) 17, Regulatory T cell (Treg) and the ratio of Th17/Treg in Follicular (upper row) and Luteal (lower row) phases in different study groups. Each box plot represents 25%-75% quartiles with the median. **: 0.001≤*p*-value≤0.05), *: *p*-value<0.001**

Comparing Frequency of Treg Cells between Three Groups

CD4+CD25+FoxP3+CD127- cells were considered as Treg cells (Figure 2). Similar to Th17 cells, Treg cell numbers were different only in the anti-TPO negative RSA group compared to fertile women in the follicular phase. In the luteal phase, these cells were upraised in the control group compared to RSA (even though with or without anti-TPO antibody). We could not detect any difference in Treg cell numbers between anti-TPO+ and anti-TPO- RSA patients in either phase (Table 2, Figure 4).

Th1/Th2, Th17/Treg Proportions

For a better interpretation of results, the proportions of Th1/Th2 and Th17/Treg cells were compared amongst different groups. These proportions were increased in both RSA groups, however, these ratios were not statistically different between two RSA groups with and without anti-TPO (Figures 3&4).

DISCUSSION

In this study, we compared the numbers of CD4+IFN-γ+, CD4+IL-4+, CD4+IL17+ and CD4+CD25+FoxP3+CD127-, which were considered as Th1, Th2, Th17, and Treg cells, in three different groups consisted of two RSA groups (anti-TPO positive and negative) and one fertile control group.

The prominent finding of our study, which was observed between anti-TPO positive and negative RSA groups, was increased numbers of Th1 cells in anti-TPO+ and this change was detected only in the luteal phase. In other words, the only CD4+ subpopulation that its frequency can be related to the presence of anti-TPO was Th1. This dominancy could be rationalized by the fact that the women who have anti-TPO, even though being euthyroid, could be at the primary stages of Hashimoto's thyroiditis and this disease represents dominantly by Th1 responses.[12,13] Several studies indicated that Th1 cells and their associated cytokines play a major role in Hashimoto's thyroiditis pathogenesis.[12,14,15] Re-emphasized the euthyroid

condition of both RSA groups, we could detect the systemic predominance of Th1 cells only in patients who had the anti-TPO antibody. This predominant Th1 response, which was observed only in the luteal phase, could result in a pro-inflammatory condition in this phase and can lead to pregnancy failure. Also, the patients in the early stages of Hashimoto's thyroiditis, who have normal thyroid function, can become hypothyroid in the early stages of pregnancy. Insufficiency of thyroid hormones in the early stages of pregnancy can cause abortion.[16] We did not observe these predominant Th1 responses in the follicular phase; however, higher numbers of Th1 cells in anti-TPO+ RSA compared to the control group in the follicular phase were near a significant level (p-value: 0.054). Maybe this non-significant difference has been due to a relatively low number of samples (four) in anti-TPO+ RSA group in follicular phase.

Contrary to Th1 cells, neither Th2, Th17, and Treg cell numbers nor Th1/Th2 and Th17/Treg proportions were different between anti-TPO positive and negative RSA groups. Previous studies suggested the upregulation of Th1 and Th17[17-20] whilst downregulation of Th2[21] and Treg cells[22,23] in Hashimoto's thyroiditis. Our results indicated that variations in CD4+T cells, except for Th1that may be related, are not associated with the pathogenesis of RSA in patients who had anti-TPO.

In the second step, we compared the frequency of different CD4+T subsets between RSA and control groups based on the menstruation phase. In the follicular phase, we could only detect the alterations of Th2, Th17, and Tregs in RSA groups in comparison with fertile women. Although in the follicular phase, upregulation of Th17 and downregulation of Treg cells were observed only in the anti-TPO negative RSA group, Th17/Treg ratios were elevated in both-anti-TPO-positive and -negative RSA groups compared to control. This finding suggests that the proportion of Th17/Treg seems to have greater value for predicting the outcome of pregnancy; regardless of the presence or absence of thyroid autoimmunity.[9] Also, in the follicular phase, decreased numbers of Th2 cells were seen in both RSA groups compared to the control group. This finding is in line with previous studies emphasized that normal pregnancy is accompanied by a deviation to Th2 responses[24,25] and decreased levels of Th2 cells could result in pregnancy complications such as RSA.[26,27]

In the luteal phase, our findings represent a preference of inflammatory Th1 and Th17 cells as major contributors to the pro-inflammatory condition which leads to RSA in anti-TPO positive as well as anti-TPO negative RSA patients. Simultaneously, we could observe the Th2 and Treg cells' reductions in RSA groups compared to fertile ones. Overall, these findings were supported by previous studies regarding predominance of Th17 cells over Treg cells[7,11,28-33] and also Th1 over Th2 cells in recurrent miscarriage.[34-40]

Besides the individual role of Th1, Th2, Th17 and Treg cells in RSA pathogenesis, we should consider that the ratios of Th1/Th2 and Th17/Treg seem to have more significance in determining the fate of pregnancy. Although we could see the deviation of these proportions in RSA groups compared to control one, this imbalance could not be detected in the comparison between RSA patients who had anti-TPO with those lacking autoantibody. These findings suggest that these ratios do not seem to be influenced by the presence of anti-TPO.

Up to our search, this study is the first one comparing the frequency of four CD4+ T subsets in RSA women with and without anti-TPO, concurrently. However, our study had some limitations. The number of menstrual phase samples differed in each group and we did not have sufficient samples based on the menstrual phase in each individual group. The second limitation was not using anti-CD3 for analyzing exclusively T cells. The third limitation of our study was not determining the anti-TPO levels in the control group.

By all of these shortcomings, our study indicates upraised Th1 cells in anti-TPO positive compared to anti-TPO negative RSA cases and this increase could be a contributing factor in the occurrence of RSA.

Overall, our results indicated that the only subset of CD4+ T cell different between RSA groups (i.e. with and without anti-TPO) was Th1 cells. Other CD4+ T cells' deviations, including Th2, Th17 and Treg cells, could be related to the presence of abortion, regardless of underlying thyroid autoimmunity state.

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