Journal of Clinical and Translational Research 2022; 8(3): 256-265

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

Evaluation of Th17 and Treg cytokines in patients with unexplained recurrent pregnancy loss

Maral Farshchi[1†], Elham Abdollahi[1,2†], Nafiseh Saghafi[2], Ahmad Hosseini[3], Sara Fallahi[4], Sirus Rostami[4], Parifar Rostami[4], Houshang Rafatpanah[1*], Mojtaba Habibagahi[3*]

[1]Immunology Research Center, Inflammation and Inflammatory Diseases Division, Mashhad University of Medical Sciences, Mashhad, Iran, [2]Department of Gynecology and Obstricts, Mashhad University of Medical Sciences, Mashhad, Iran, [3]Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran, [4]Shiraz IVF Center, Shiraz University of Medical Sciences, Shiraz, Iran

[†]These authors contributed equally to this work

ARTICLE INFO

Article history:
Received: May 18, 2021
Revised: November 28, 2021
Accepted: April 07, 2022
Published online: May 25, 2022

Keywords:
immunological responses
T helper 17 cells
Tregs
unexplained recurrent pregnancy loss

**Corresponding authors:*
Houshang Rafatpanah
Immunology Research Center, Inflammation and Inflammatory Diseases Division, Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran.
E-mail: Rafatpanahh@mums.ac.ir
Mojtaba Habibagahi
Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran.
E-mail: habibagahim@yahoo.com

ABSTRACT

Background and Aim: The Th17/Treg balance in peripheral blood and reproductive tissues may have a role in the etiology of unexplained recurrent pregnancy loss (URPL). In this study, we evaluated the major cytokine of Treg cells transforming growth factor-beta (TGF-β), and their specific transcription factor Forkhead box P3 (FOXP3), as well as the most highlighted cytokine of Th17 cells (interleukin [IL]-17A) in both URPL patients and healthy women.

Methods: Samples were extracted from the peripheral blood, endocervix, endometrium, and vagina of 14 patients with URPL and 12 normal non-pregnant women as a control (normal) group. Quantitative reverse transcription polymerase chain reaction was used to determine gene expression. Enzyme-linked immunosorbent assay was used to determine the levels of cytokines in the serum and cervicovaginal fluid.

Results: We found that in the URPL group, FOXP3 gene expression was considerably higher in peripheral blood than in the normal group (P=0.043). TGF-β levels in the cervicovaginal fluid were different in the URPL and normal groups (P=0.015). In comparison to the control group, women with URPL had significantly greater IL-17 gene expression in the peripheral blood (P=0.01).

Conclusion: Lower TGF-β levels in the cervicovaginal fluid of patients compared to controls may be related with increased IL-17 and FOXP3 mRNA levels in patients with URPL. Dysregulation of local immune responses in reproductive tissues may represent dysregulation of systemic regulatory immunological responses in the pathogenesis of URPL.

Relevance for Patients: Dysregulation of immune responses may play a role in the pathogenesis of URPL at least in some patients with URPL. We conclude that the breakdown of tolerance in the local immune responses is more critical than the breakdown of tolerance in systemic tolerance in the pathogenesis of URPL. Therefore, modulating immune responses in the endometrium and decidua may be the focus of future therapeutic approaches in URPL. The impact of seminal plasma on the expansion of Tregs may provide a novel therapeutic intervention that has already been used in assisted reproductive technologies. Therefore, we suggest that transvaginal TGF-β in women with URPL may induce maternal tolerance which leads to the successful pregnancy.

1. Introduction

The fetus is a semi-allogeneic graft, with half of its MHC molecules coming from the mother and half from the father [1,2]. As a result, the fetus is antigenic while the mother is immunologically responsive. The mother's immune system must tolerate the fetus during

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

pregnancy, but if this tolerance is lost, recurrent pregnancy loss or spontaneous abortion may occur [1,3]. Recurrent pregnancy loss (RPL) is defined as three or more recurrent losses before the 20th week (or first trimester of pregnancy) of pregnancy [4,5]. RPL affects about 1–5% of women [6,7].

Although RPL could be caused by a variety of factors including genetic, endocrine, anatomic, and viral agents, the etiology of approximately 50% of RPL cases is unclear, which is referred to as unexplained RBL (URPL) [8].

URPL is defined as three or more spontaneous sequential miscarriages before 20 weeks of pregnancy without a known cause [9]. In this view, deregulation of immune system is highly probable to be contributed to the pathogenesis of URPL. Mounting evidence indicated that there is a strong correlation between URPL and the failure of maternal immunologic tolerance [10].

T helper 1 (Th1), Th2, regulatory T cells (Treg cells), and Th17 cells are subsets of $CD4^+$ T cells that play an important role in the maternal immune responses [4,11-14]. Maternal tolerance toward fetal alloantigens was explained by the predominant Th2-type immunity during pregnancy, while a predominant Th1-type immune response has been observed in recurrent spontaneous abortion [12,15]. More recently the emerging concept of the balance of Th17/Treg has challenged the conventional paradigm of the Th1/Th2 hypothesis [16,17]. Treg cells ($CD4^+$ $CD25^+$ high Forkhead box P3 [$FOXP3^+$]) expressing CTLA-4 are necessary to mediate maternal tolerance to the fetus [15,18-21]. FOXP3 is a master transcription factor of Treg cells which is necessary for the function of those cells [10,22].

Treg cells contribute to a successful pregnancy by suppressing self-reactive lymphocytes. Treg cells act through cell-cell contact (mediated by the negative regulators of T-cell activation such as CTLA-4) and by releasing of the anti-inflammatory cytokines such as transforming growth factor-beta (TGF-β) and interleukin (IL-10) [23-30].

TGF-β is a multifunctional cytokine released by a variety of immune cells, including peripheral blood mononuclear cells (PBMCs) and Treg cells, that have been shown to play a role in both promoting and inhibiting placental development [4,31]. TGF-β has been shown to maintain peripheral natural nTregs that develop in the thymus and to induce the differentiation of naive $CD4^+$ T cells to iTregs. Interestingly, TGF-β is also important in differentiation of Th17 cells Th17 cells differentiate from naive $CD4^+$ T cells in the presence of TGF-β and IL-6 [19,32]. It was found that a high dose of TGF-β and an intermediate dose of IL-10 suppressed the release of IL-17 [19,33].

Th17 ($CD4^+$ $IL\text{-}17A^+$) is characterized by the expression of IL-17A, IL-17 F, IL-21, IL-22, IL-6, and TNF-α [34-38]. Th17 cells contribute to host defense against both extracellular pathogens and as well as fungal infections. Excessive inflammation and oxidative stress are important in the pathogenesis of pregnancy disorders [39]. In humans, IL-17 has been involved in the development and progression of inflammatory and autoimmune diseases, as well as transplant rejection [36,40-43]. Th17 cells are regulated by Treg cells, which play a significant role in the establishment and maintenance of tolerance [37,44,45]. Th17 cells are related to the development of inflammation in late stage of abortions [46-49].

Several studies evaluated the Th17/Treg balance in peripheral blood from women with URPL, and it was demonstrated that local immune responses play a unique role in URPL pathogenesis [25,46,48,50-61].

This study aimed to determine IL17A, TGF-β, and FOXP3 gene expression in peripheral blood, vaginal, endometrium, and cervical cells. Furthermore, TGF-β and IL-17A serum levels and cervicovaginal levels of TGF-β and IL-17A in URPL patients and healthy women were measured.

2. Materials and Methods

2.1. Patients and controls

This case–control study was conducted from 2010 to 2012 on 14 non-pregnant women with URPL and 12 fertile non-pregnant women. Fertile non-pregnant women with a history of normal deliveries (control group) who were referred to Shiraz Health Center, Iran, had no history of spontaneous abortion, ectopic pregnancy, and preterm delivery.

To determine the sample size, we used a case–control study related to unexplained infertility conducted by Jasper *et al*. [62]. The sample size for the URPL group (n=10) and the control group (n=12) was selected based on FOXP3 gene expression [62].

Finally, using the formula below and considering the dropout of patients, the sample size 14 was calculated for the URPL group (CI=95%, power=80%).

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2[S_1^2 + S_2^2]}{(\bar{X}_1 - \bar{X}_2)}$$

For all patients and controls, it had been at least 3 months from their last miscarriage or successful pregnancy. The women in the URPL group did not have any other known medical conditions apart from the RPL and were not under any medication. Male partners underwent semen analyses. The number; shape, and movement of sperm were measured and were found to be normal. Both URPL and control groups were at reproductive age with regular menstruation, a normal BMI, not pregnant as confirmed by a negative blood human chronic gonadotropin test, and were without any uterine, cervical, or genetic abnormalities. Women in the URPL group were excluded from the present study if they had a positive screening test such as hormone tests, Treponema pallidum particle agglutination assay, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, male and female karyotypes, antinuclear antibodies, anti-cardiolipin antibodies, and lupus anticoagulant antibodies antiphospholipid antibodies, fewer than three consecutive miscarriages. Inclusion criteria were a normal result in the routine mentioned laboratory test panel. Inclusion criteria for the controls were to have no history of abortion (at least have one healthy baby) with normal results in routine laboratory tests (as mentioned above). The mean age of patients was 29.8±4.4 year and the mean age of the controls was 30.5±5.6 (P=0.40). Clinical characteristics of subjects are presented in Table 1.

The study was approved by the Research Ethics Committee of Shiraz University of Medical Sciences and samples were collected only from patients who had given informed consent.

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

2.2. Sample collection

All of the women in the study had menstrual cycles that ranged between 26 and 31 days. In general, women were in the secretory phase (during which progesterone hormone is secreted by the ovaries) during the final 14 days of their monthly cycles, whereas the proliferative phase was recognized before the last 14 days of the menstrual cycles [63]. This might be explained by the potential of progesterone in boosting Treg cells proliferation [64]. Thus, we chose all patients according to their last menstrual period on days 17–23 (during the secretory phase) of the menstrual cycle, with confirmation by routine histological dating. Women who were menstruating or in the proliferative phase were excluded from the study.

Peripheral blood cells were collected from all subjects. Samples from vaginal, endocervical cells, endometrial cells, as well as the cervicovaginal fluid of all women were collected by Cytobrush Plus and endometrial cell sampler, respectively. To prevent samples from drying, 50 µL of EDTA was added to all microtubes containing endometrial cells and 300 µL PBS in microtubes containing vaginal and endocervical cells. PBMCs and cervical samples after aliquoting were stored at −70°C until they were ready to be analyzed.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Serum levels of IL-17A and TGF-β, and sex hormones (E2, progesterone, prolactin, follicle-stimulating hormone, and luteinizing hormone) were measured by ELISA (eBioscience, Austria), according to the manufacturer's instructions.

Table 1. Clinical characteristics of the cohort

	Control (*n*=12)	Patients with unexplained recurrent pregnancy loss (*n*=14)
Age (years)	29.8±4.4	30.5±5.6
Body mass index (BMI)	23.3±5.9	24.5±6.0
Mean number of miscarriages	4 (range=3–7 miscarriages)	-
Mean time of miscarriage (week of gestation)	5.5	-
Mean of time of last miscarriage (months passed from last miscarriage)	8.2	-
TPPA	Negative	
HIV	Negative	
HBV	Negative	
HCV	Negative	
Anti-cardiolipin antibodies	Negative	
Lupus anticoagulant antibodies	Negative	
Anti-phospholipid antibodies	Negative	
B-hCG		

TPPA: Treponema pallidum particle agglutination assay; HIV: Human immunodeficiency virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; B-hCG: Human chronic gonadotropin

2.4. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

RNA extraction kit (Invitek, Germany) was used for total RNA extraction from PBMCs as per manufacturer's instructions. Reverse transcriptions were performed by RevertAid H primers (Germany). Primer-BLAST was used to ensure that primers were specific. qPCR master mix (SYBR Green, Takara) was used to run the specified primers at varying concentrations (2, 5, and 10 pmoles/uL) using control cDNA (25 ng/uL, OriGene). Each concentration was run in triplicates. The melt curve peaks and amplification plots were then checked. In the same qPCR experiment, a control was run without any samples and just primer and master mix were used to evaluate primer dimers and their Tm on a melt curve plot. The product melt curves were compared with these temperatures which were significantly lower than the products. The products were run on a 2.5% agarose gel, and the band sizes of the products were compared [65,66]. The primers used in our study produced precise product bands.

RNA quality was confirmed by agarose gel (2%) electrophoresis using 5.8S, 18S, and 28S bands as revealed by a UV light transilluminator. To estimate nucleic acid purity, the ratio of the absorbance contributed by the nucleic acid to the absorbance of the contaminants was measured. A_{260}/A_{280} ratios were 1.9–2.1.

cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific). The total volume of all PCR reactions was 20 µL containing 10 µL of Real-time PCR-SYBR Green Master Mix (Takara, Japan), 0.3 µL of each primer (Table 1), and 7.4 µL of RNase-free water. Real-time PCR was performed by Rotor-Gene Q cycler (Qiagen, Germany). The following standard PCR reaction conditions were used for all transcripts: 10 min at 95°C, 15 s at 95°C (45 cycles), 30 s at 57°C, and 1 min at 60°C.

Logarithmic dilution series of the total RNA was used to construct 10-fold dilution standard curves for FOXP3, IL-17A, and TGF-β. 18s rRNA was used as the internal control gene to normalize mRNA levels between the mentioned cytokines. Data were analyzed using Rotor-Gene 6000 software (Qiagen, Germany) to quantify the cDNA copy number for each gene. The relative quantity of gene of interest was normalized to the relative quantity of 18s rRNA and was reported as a fold change of gene expression.

The sequences of the primers and probes are shown in Table 2.

2.5. Serum collection procedure

After collection of the whole blood (3 mL), the blood was allowed to clot at room temperature (15–30 min). The clot was removed by centrifuging at 1000–2000×*g* for 10 min in a cooled centrifuge. The supernatant as the serum was collected for measuring serum levels of sex hormones (Table 3). The serum levels of sex hormones were measured using the chemiluminescence method.

2.6. Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 software and GraphPad Prism 7.0. Data are presented as mean±SD. Cytokine expression between all groups was performed using the

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

nonparametric Kruskal–Wallis test. The Mann–Whitney U-test was applied for analyzing the differences between URPL and control groups. $P \leq 0.05$ was considered statistically significant.

3. Results

There was no significant difference between the mean serum levels of sexual hormones between URPL and the control groups (Table 2).

3.1. FOXP3 gene expression in patients with URPL and the controls

The results indicated that FOXP3 gene expression was 2.2-fold higher in peripheral blood of patients with URPL compared to the controls (P=0.043). FOXP3 gene expression in vaginal and endometrial cells from patients with URPL increased, while this change was not significant (2.3 and 2.4, P>0.05). The mean FOXP3 gene expression in endocervical cells in the control and patient groups did not differ significantly (P>0.05) (Figure 1).

3.2. TGF-β gene expression in patients with URPL and the controls

TGF-β gene expression was 2.5 times higher in the peripheral blood of URPL patients than in healthy women, which was not statistically significant (P>0.05) (Figure 2).

Table 2. Primer and probe sequences used in quantitative RT-PCR

Targeted gene	Sequence (5' to 3')		Product size (bp)
FOXP3	CACCTGGAAGAACGCCATCC	Forward	94
	CTCATCCACGGTCCACACAG	Reverse	
	CCCTTCTCGCTCTCCACCCGCACA	Probe	
	TCACCTGCTCCACGGCCTTGCTCT	Probe	
IL-17A	GGAATCTCCACCGCAATGAGG	Forward	130
	TGCTGGATGGGGACAGAGTTC	Reverse	
	TGGTAGTCCACGTTCCCATCAGCG	Probe	
TGF-β	CCAGAAATACAGCAACAATTCCTG	Forward	136
	GCCCTCAATTTCCCCTCCAC	Reverse	
	CTCAACCACTGCCGCACAACTCCG	Probe	
18s rRNA	GTTGATTAAAGTCCCTGCCCTTTG	Forward	152
	TCCGAGGGCCTCACTAAACC	Reverse	
	ACACACCGCCCGTCGCTACACCG	Probe	

RT-PCR: Reverse transcription polymerase chain reaction; FOXP3: Forkhead box P3, IL-10: Interleukin 10; TGF-β: Transforming growth factor-beta

Table 3. Sex hormone levels of the URPL and control group

Hormone	URPL group n=14	Control group n=12	P-value
E2 (pg/mL)	207.8±211.5	222.8±166	0.368
Progesterone (ng/mL)	5.9±7.8	5.7±6.4	0.699
Prolactin (ng/mL)	21.7±25.8	18.2±22.6	0.269
FSH (mIU/mL)	5.3±4.8	4.0±2.4	0.758
LH (mIU/mL)	15.1±19.0	14.7±19.3	0.738
LH/FSH ratio	2.4±1.2	3.2±2.0	0.224

LH: Luteinizing hormone, FSH: Follicle-stimulating hormone, URPL: Unexplained recurrent pregnancy loss

3.3. TGF-β cervicovaginal fluid levels in patients with URPL and the controls

The levels of cervicovaginal fluid TGF-β in the URPL group were significantly lower than that in the control group (27.5±3.6 pg/mL vs. 30.8±2.9 pg/mL, P=0.015). In contrast, TGF-β serum levels were not statistically different between URPL and control groups (104.1±45.8 pg/mL vs. 75.8±45.1 pg/mL, P>0.05) (Figure 3).

3.4. IL-17 gene expression in patients with URPL and the controls

The expression of the IL-17 gene was higher in the peripheral blood of patients with URPL compared to the control group (P=0.01). IL-17 gene expression in endocervical and endometrial cells was higher (4.4- and 13.6-fold) than that in the control group, however, this difference was not significant (P>0.05). In both groups, IL-17 gene expression in vaginal cells was statistically similar (P>0.05) (Figure 4).

3.5. IL-17 serum levels in patients with URPL and the controls

There was no significant difference between IL-17 serum levels and IL-17 cervicovaginal levels in control and URPL groups (4.1±3.8 pg/mL vs. 3.5±1.9 pg/mL, P>0.05, 1.8±7.2 pg/mL vs. 0.9±2.2 pg/mL, P>0.05).

3.6. Comparison of sexual intercourse between URPL and control groups

The sexual intercourse in patients with URPL was higher than that in the control group. The time interval of sexual contact

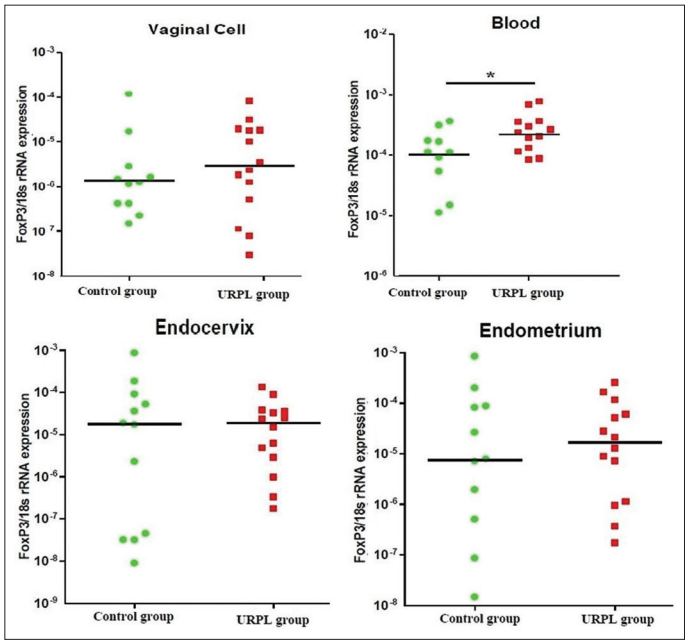

Figure 1. Forkhead box P3 (FOXP3) gene expression. FOXP3 gene expression in peripheral blood in the unexplained recurrent pregnancy loss (URPL) group was significantly higher compared to the control group. FOXP3 gene expression in other source material was comparable to the control group. The results show $2^{-\Delta CT}$ gene expression. The mean is indicated with a line. *Represents P<0.05.

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

was lower in the URPL group than that in the control group (Figure 5).

4. Discussion

The following are the main findings of the current study: First, in women with URPL, FOXP3 gene expression was shown to be considerably higher in the peripheral blood. Second, compared to the control group, TGF-β levels in cervicovaginal secretions were considerably lower in patients with URPL. Third, IL-17 gene expression in the peripheral blood of women with URPL was considerably higher than that in the control group.

Circulating $CD4^+$ $CD25^+$ Treg cells were reported to increase during early stages of pregnancy and then decline after postpartum [55]. Previous research demonstrated that Treg cells from maternal peripheral blood were recruited to the fetal-maternal interface (endometrium and decidua) at the first trimester of pregnancy, indicating local immune regulation against fetus-specific antigens [50,67-69].

Treg cells impairment was found in women with both URPL and sporadic abortions. This may explain why women with URPL have a higher percentage of Treg cells than normal women. This leads to suppress $CD4^+$ effector cell proliferation in response to paternal alloantigens [70]. We also indicated the lower percentage of Treg cells and the lower FOXP3 gene expression in patients with URPL compared to the controls [71,72]. In the present study, we analyzed the expression of the FOXP3 gene in peripheral

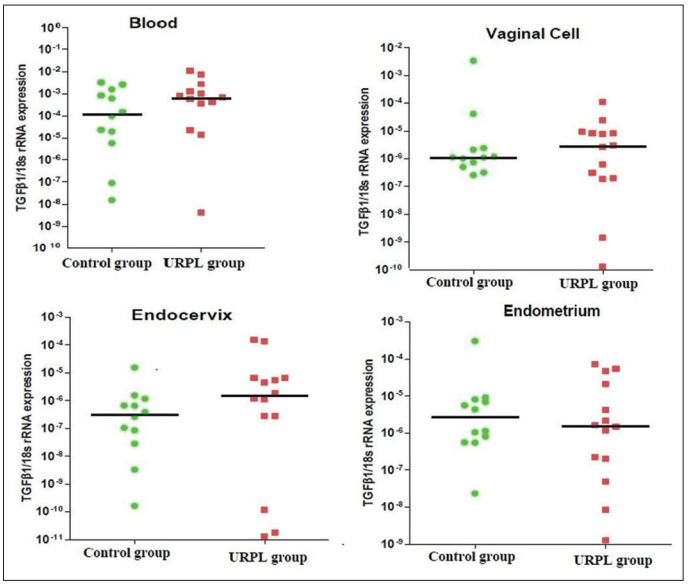

Figure 2. Transforming growth factor (TGF)-β gene expression. TGF-β gene expression in peripheral blood, vaginal, endometrial, and endocervical cells in patients with URPL was not significantly different compared to the control group. The mean is indicated with a line.

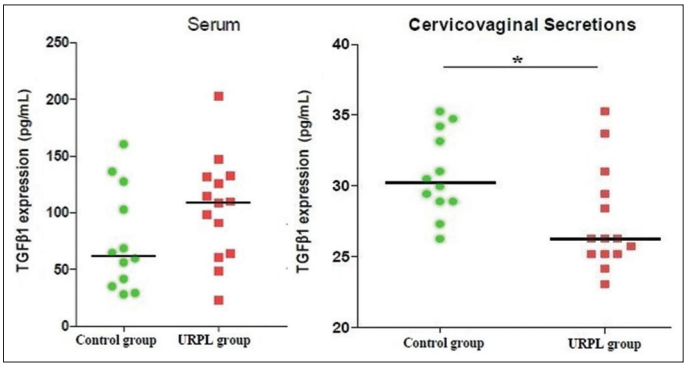

Figure 3. Transforming growth factor (TGF)-β expression in the serum and cervicovaginal secretions. TGF-β cervicovaginal levels are lower in the unexplained recurrent pregnancy loss group compared to the control group. The mean is indicated with a line. *Represents $P<0.05$.

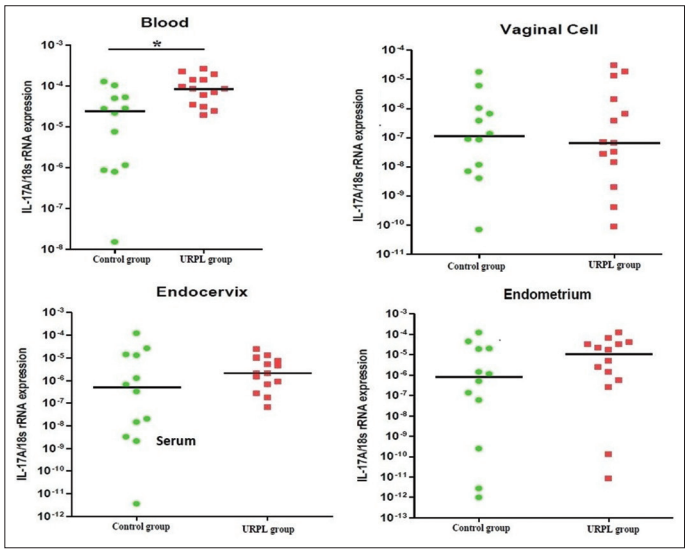

Figure 4. Interleukin (IL)-17A gene expression. IL-17 gene expression was only higher in peripheral blood of women with unexplained recurrent pregnancy loss compared to the control group. The mean is indicated with a line. *Represents $P<0.05$.

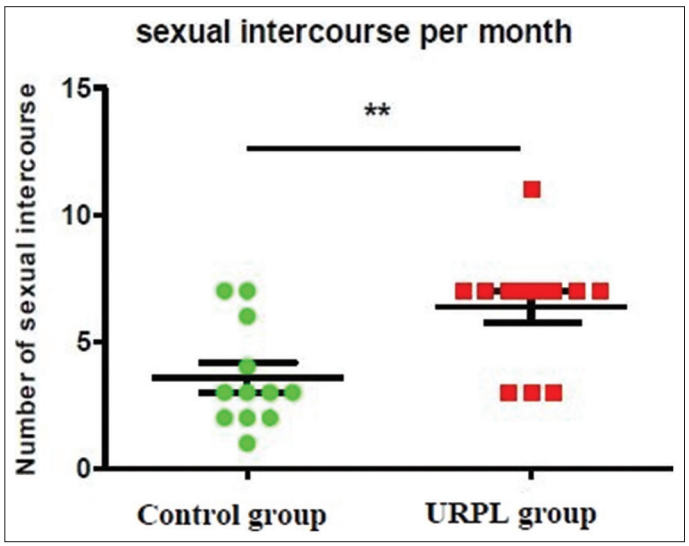

Figure 5. Sexual intercourse frequency. The mean is indicated with a line. **Indicates $P<0.01$.

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

blood, endocervix, endometrium, and vaginal cells. We showed that the expression of the FOXP3 gene in Treg cells increased. As mentioned, Treg cells from women with URPL may be functionally deficient, therefore, FOXP3 gene expression may increase as the manner of compensating in response to Treg cells impairment in patients with URPL [73-75].

Furthermore, it was found that the frequency of single-nucleotide polymorphisms including, rs2232365, rs3761548, rs5902434, and rs2294021 was increased in women with URPL compared to the control women [76,77]. It was suggested that the functional polymorphisms of the FOXP3 gene may contribute to alter FOXP3 gene expression in patients with URPL [78]. As a result, the variation of the FOXP3 gene that may impact its expression in women with URPL should be considered. Furthermore, in patients with URPL, the number and length of sexual encounters were significantly higher than in the control group. Consequently, we suggest that FOXP3 gene expression may be increased in response to paternal alloantigens.

TGF-β is a multifunctional cytokine that is secreted by several types of immune cells including Treg cells and Th2 cells. TGF-β was found to induce the differentiation of naive $CD4^+$ T cells to iTregs. Interestingly, TGF-β also is important in Th17 differentiation. In the presence of TGF-β and IL-6, naive $CD4^+$ T cells differentiate into Th17 cells [19]. It was demonstrated that TGF-β may enhance the function of Treg cells *ex vivo* [53,79-81]. In addition, TGF-β deficiency eliminated the suppressive activities of Treg cells *in vivo* [79]. These findings suggested that the immunosuppressive activity of Treg cells depends on TGF-β [82,83]. TGF-β is important in female reproduction, and dysregulation of the TGF-β signaling pathway may lead to reproductive complications [81]. It was shown that decreased levels of tolerogenic cytokines (IL-10 and TGF-β) in the endometrium were the potential pathogenic mechanism leading to URPL occurrence [84]. Our study showed that TGF-β gene expression in peripheral blood, vaginal cells, and endocervical cells in patients with URPL partially increased compared to the control group. In contrast, TGF-β levels in cervicovaginal secretions were significantly lower in patients with URPL compared to the control group.

Surprisingly, while the number of sexual encounters was higher in URPL patients, TGF-β levels in cervical secretion were lower in those patients compared to the healthy women. Regarding the involvement of TGF-β in the regulation of immune responses, this study suggests that dysregulation of local immune responses in the uterus may be more important compared to the systemic immune responses in the pathogenesis of URPL. Corroboratively, a study in a mouse model of abortion indicated that transvaginal pure TGF-β promoted Treg cells and their requirement to the vagina resulted in a reduction in the rate of abortion. Th17 cells that produce IL-17 (as the pro-inflammatory cytokine) may play a key role in the immunopathogenesis of URPL [12,16,25,85,86]. The proportions of Th17 cells in both peripheral blood and decidua as well as expression of IL-17A on $CD4^+$ T cells were found significantly higher in URPL than in the normal pregnant women [50,61,87,88].

We showed the increased frequency of Th17 cells along with the decrease in the percentage of Treg cells in URPL patients [56,57,72,89,90]. The present study also showed that IL-17 gene expression significantly increased in peripheral blood in women with URPL.

In summary, the present study indicated a significant decrease in cervical TGF-β levels that were associated with a considerable increase in IL-17A serum levels in women with URPL. In this regard, we propose that deregulation of local immune responses may play a role in the etiology of URPL.

4.1. Clinical implications

Dysregulation of immune responses may play a role in the pathogenesis of URPL at least in some patients with URPL [91]. Modulating immune responses in the endometrium and decidua may be the focus of future therapeutic approaches in URPL. For example, the impact of seminal plasma on the expansion of Tregs may provide a novel therapeutic intervention that has already been used in assisted reproductive technologies [92-94]. TGF-β is a major component of seminal plasma that plays a role in regulating the maternal immune response [92]. According to our findings, levels of TGF-β in cervicovaginal fluid were lower in women with URPL compared to the control group. Therefore, we suggest that transvaginal TGF-β in women with URPL may induce maternal tolerance which leads to the successful pregnancy.

4.2. Research implications

In this study, the gene expressions and protein levels of the highlighted markers and cytokines of Th17 and Treg cells were evaluated in peripheral blood, vagina, endocervix, and endometrium in women with URPL and the controls. In the future studies, the TGF-β signaling pathways in the uterus should be evaluated. To develop new therapeutic approaches in URLP treatment, we also suggest that further researches should be conducted on homing receptors, integrin, and chemokines that are involved in immune homeostasis in the uterus and decidua.

4.3. Strengths and limitations

The small sample size was a limitation of this study, therefore, correlation could not be viewed as causation. We suggest that larger patient cohort studies are needed to confirm the findings and conclusions.

It is technically not feasible to collect the decidual samples immediately after the demise of the fetus and the immune status is not in the typical patterns during the abortion. We determined the cytokine levels both in the peripheral blood and the cervicovaginal fluid from women with URPL and the controls. Therefore, the present study evaluated the local and systemic immune responses in URPL. Indeed, peripheral blood cells alone are not the most relevant place to study the immunological changes in pregnancy [63]. The major merit of the study is to perform experiments on tissues involved in pregnancy outcomes.

5. Conclusion and Future Directions

We found that the decreased levels of TGF-β in the secretions of cervicovaginal may increase the expression of the IL-17 gene in

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

peripheral blood in patients with URPL. Therefore, we conclude that the breakdown of tolerance in the local immune responses is more critical than the breakdown of tolerance in systemic tolerance in the pathogenesis of URPL. We suggest that targeting local immune responses may be an appropriate approach in the treatment of URPL.

Acknowledgments

We would like to thank Dr Zaleh Zolghadr for clinical examination of patients and their referral for participation in the study; alongside Dr Mansoureh Jaberipour for her scientific advice thought the duration of the study. We would also like to thank the Clinical Research Development Unit, Qaem Hospital and the Vice Chancellor of Mashhad and Shiraz University of Medical Sciences, for financial support and the staff of the Shiraz IVF Center for their nice cooperation in this study.

Conflicts of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Details of Ethics Approval

The study was approved by the Research Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.sm.REC.1392.196) and samples were collected only from patients who had given informed consent.

References

[1] Heitmann RJ, Weitzel RP, Feng Y, Segars JH, Tisdale JF, Wolff EF. Maternal T Regulatory Cell Depletion Impairs Embryo Implantation Which Can Be Corrected With Adoptive T Regulatory Cell Transfer. Reprod Sci 2016;4:1014-24.

[2] Deng T, Li J, Li X, Li X, Yan Y. The Effects of Isolates and Immune Function on Hematologic Parameters of Blastocystis Infection Rats. Trends Immunother 2021;5:1-7.

[3] Robertson SA, Petroff MG, Hunt JS. Immunology of Pregnancy. In: Knobil and Neill's Physiology of Reproduction. 4th ed. Oxford, UK: Elsevier; 2015. p. 1835-74.

[4] Barad DH, Kushnir VA, Gleicher N. Focus on Recurrent Miscarriage Phenotypes. Fertil Steril 2017;107:64.

[5] Lee SK, Na BJ, Kim JY, Hur SE, Lee M, Gilman-Sachs A, *et al*. Determination of Clinical Cellular Immune Markers in Women with Recurrent Pregnancy Loss. Am J Reprod Immunol 2013;70:398-411.

[6] Rai R, Regan L. Recurrent Miscarriage. Lancet 2006;368:601-11.

[7] Arjmand F, Ghasemi N, Mirghanizadeh SA, Samadi M. The Balance of the Immune System between HLA-G and NK Cells in Unexplained Recurrent Spontaneous Abortion and Polymorphisms Analysis. Immunol Res 2016;64:785-90.

[8] Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T Cells Mediate Maternal Tolerance to the Fetus. Nat Immunol 2004;5:266.

[9] Roepke ER, Bruno V, Nedstrand E, Boij R, Strid CP, Piccione E, *et al*. Low-molecular-weight-heparin Increases Th1-and Th17-associated Chemokine Levels during Pregnancy in Women with Unexplained Recurrent Pregnancy Loss: A Randomised Controlled Trial. Sci Rep 2019;9:1-10.

[10] La Rocca C, Carbone F, Longobardi S, Matarese G. The Immunology of Pregnancy: Regulatory T Cells Control Maternal Immune Tolerance Toward the Fetus. Immunol Lett 2014;162:41-8.

[11] Cyprian FS, Lefkou E, Varoudi A, Girardi G. Immunomodulatory Effects of Vitamin D in Pregnancy and Beyond. Front Immunol 2019;10:2739.

[12] Pei CZ, Kim YJ, Baek KH. Pathogenetic Factors Involved in Recurrent Pregnancy Loss from Multiple Aspects. Obstet Gynecol Sci 2019;62:212-23.

[13] Ghaneifar Z, Abdollahi E. Evaluation of the Effects of 1, 25VitD3 on Inflammatory Responses and IL-25 Expression. Front Genet 2021;12:779494.

[14] Abdollahi E, Momtazi AA, Johnston TP, Sahebkar A. Therapeutic Effects of Curcumin in Inflammatory and Immune-mediated Diseases: A Nature-made Jack-of-all-trades? J Cell Physiol 2018;233:830-48.

[15] Tsuda S, Nakashima A, Shima T, Saito S. New Paradigm in the Role of Regulatory T Cells during Pregnancy. Front Immunol 2019;10:573.

[16] Abdollahi E, Tavasolian F, Momtazi-Borojeni AA, Samadi M, Rafatpanah H. Protective Role of R381Q (rs11209026) Polymorphism in IL-23R Gene in Immune-mediated Diseases: A Comprehensive Review. J Immunotoxicol 2016;13:286-300.

[17] Kanannejad Z, Jahromi BN, Gharesi-Fard B. T Cell Subsets Profiling in Unexplained Infertile Women with Successful and Unsuccessful *in Vitro* Fertilization Outcome: Focus on the Effect of Seminal Plasma. Iran J Allergy Asthma Immunol 2019;18:163-72.

[18] Teles A, Thuere C, Wafula PO, El-Mousleh T, Zenclussen ML, Zenclussen AC. Origin of Foxp3 (+) Cells during Pregnancy. Am J Clin Exp Immunol 2013;2:222-33.

[19] Care AS, Bourque SL, Morton JS, Hjartarson EP, Robertson SA, Davidge ST. Reduction in Regulatory T Cells in Early Pregnancy Causes Uterine Artery Dysfunction in Mice. Hypertension 2018;72:177-87.

[20] Mohammadi S, Abdollahi E, Nezamnia M, Esmaeili SA, Tavasolian F, Sathyapalan T, *et al*. Adoptive Transfer of Tregs: A Novel Strategy for Cell-based Immunotherapy in Spontaneous Abortion: Lessons from Experimental Models. Int Immunopharmacol 2021;90:107195.

[21] Nian D, Li Z, Sun J, Shi P. Effect of 1.25 (OH) 2D3 on Experimental Autoimmune Neuritis and its Mechanism.

Trends Immunother 2021;5:42-50.

[22] Fontenot JD, Gavin MA, Rudensky AY. Foxp3 Programs the Development and Function of CD4+ CD25+ Regulatory T Cells. Nat Immunol 2003;4:330.

[23] Corthay A. How do Regulatory T Cells Work? Scand J Immunol 2009;70:326-36.

[24] Vignali DA, Collison LW, Workman CJ. How Regulatory T Cells Work. Nat Rev Immunol 2008;8:523-32.

[25] Hosseini S, Shokri F, Pour SA, Jeddi-Tehrani M, Nikoo S, Yousefi M, *et al*. A Shift in the Balance of T17 and Treg Cells in Menstrual Blood of Women with Unexplained Recurrent Spontaneous Abortion. J Reprod Immunol 2016;116:13-22.

[26] Deshmukh H, Way SS. Immunological Basis for Recurrent Fetal Loss and Pregnancy Complications. Ann Rev Pathol Mech Dis 2019;14:185-210.

[27] Field EH, Kulhankova K, Nasr ME. Natural Tregs, CD4+ CD25+ Inhibitory Hybridomas, and their Cell Contact Dependent Suppression. Immunol Res 2007;39:62-78.

[28] Yang Z, Xu Y, Bi Y, Zhang N, Wang H, Xing T, *et al*. Immune Escape Mechanisms and Immunotherapy of Urothelial Bladder Cancer. J Clin Transl Res 2021;7:485.

[29] Gutierrez SD, da Silva Aguiar S, Barbosa LP, Santos PA, Maciel LA, de Araújo Leite PL, *et al*. Is Lifelong Endurance Training Associated with Maintaining Levels of Testosterone, Interleukin-10, and Body fat in Middle-aged Males? J Clin Transl Res 2021;7:450.

[30] Farrugia M, Baron B. The Role of TNF-α in Rheumatoid Arthritis: A Focus on Regulatory T Cells. J Clin Transl Res 2016;2:84.

[31] Yang F, Zheng Q, Jin L. Dynamic Function and Composition Changes of Immune Cells during Normal and Pathological Pregnancy at the Maternal-fetal Interface. Front Immunol 2019;10:2317.

[32] Inaba Y, Kunimoto K, Kanazawa N, Furukawa F. Effects of a Humanized Anti-human IL-6 Receptor Monoclonal Antibody on Nakajo-Nishimura Syndrome. Trends Immunother 2018;2:237.

[33] Ingman WV, Robertson SA. Defining the Actions of Transforming Growth Factor Beta in Reproduction. Bioessays 2002;24:904-14.

[34] Takci Z, Tekin Ö, Ertuğrul DT, Karadağ AS, Akin KO. A Case-control Study: Evaluation of Vitamin D Metabolism in Patients with Vitiligo. Turk J Med Sci 2015;45:837-41.

[35] Chen Z, O'Shea JJ. Th17 Cells: A New Fate for Differentiating Helper T Cells. Immunol Res 2008;41:87-102.

[36] Rengarajan T, Keerthiga S, Duraikannu S, Periyannan V. Exploring the Anticancer and Anti-inflammatory Activities of Ferruginol in MCF-7 Breast Cancer Cells. Cancer Plus 2020;1:106.

[37] Poles J, Karhu E, McGill M, McDaniel HR, Lewis JE. The Effects of Twenty-four Nutrients and Phytonutrients on Immune System Function and Inflammation: A Narrative Review. J Clin Transl Res 2021;7:333.

[38] Olthof PB, van Golen RF, Reiniers MJ, Kos M, Alles LK, Maas MA, *et al*. IL-23 and IL-17A are Not Involved in Hepatic/Ischemia Reperfusion Injury in Mouse and Man. J Clin Transl Res 2015;1:180.

[39] Ghaneifar Z, Yousefi Z, Tajik F, Nikfar B, Ghalibafan F, Abdollahi E, *et al*. The Potential Therapeutic Effects of Curcumin on Pregnancy Complications: Novel Insights into Reproductive Medicine. IUBMB Life 2020;72:2572-83.

[40] Miossec P, Kolls JK. Targeting IL-17 and TH17 Cells in Chronic Inflammation. Nat Rev Drug Discov 2012;11:763-76.

[41] Abdollahi E, Tavasolian F, Samadi M. Relationship between High-density Lipoprotein and C-reactive Protein in Patients with Atherosclerosis. Med Lab J 2014;8:97-102.

[42] Abdollahi EB, Tavasolian FB, Vakili M, Samadi M. Evaluation of Serum CRP and Vitamin D in Rheumatoid Arteritis Patients and Healthy People. Med Lab J 2015;9:90-6.

[43] Balaji S, Cholan PK, Victor DJ. An Emphasis of T-cell Subsets as Regulators of Periodontal Health and Disease. J Clin Transl Res 2021;7:648.

[44] Noack M, Miossec P. Th17 and Regulatory T Cell Balance in Autoimmune and Inflammatory Diseases. Autoimmun Rev 2014;13:668-77.

[45] Ghaedi N, Forouhari S, Zolghadri J, Sayadi M, Nematollahi A, Khademi K. Vitamin D Deficiency and Recurrent Pregnancy Loss in Iranian Women. Global Adv Res J Med Med Sci 2016;5:194-8.

[46] Nakashima A, Ito M, Shima T, Bac ND, Hidaka T, Saito S. Accumulation of IL-17-positive Cells in Decidua of Inevitable Abortion Cases. Am J Reprod Immunol 2010;64:4-11.

[47] Liu F, Guo J, Tian T, Wang H, Dong F, Huang H, *et al*. Placental Trophoblasts Shifted Th1/Th2 Balance Toward Th2 and Inhibited Th17 Immunity at Fetomaternal Interface. Apmis 2011;119:597-604.

[48] Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, *et al*. The 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D from the Institute of Medicine: What Clinicians Need to Know. J Clin Endocrinol Metab 2011;96:53-8.

[49] Li N, Saghafi N, Ghaneifar Z, Rezaee SA, Rafatpanah H, Abdollahi E. Evaluation of the Effects of 1, 25VitD3 on Inflammatory Responses and IL-25 Expression. Front Genet 2021;12:779494.

[50] Liu YS, Wu L, Tong XH, Wu LM, He GP, Zhou GX, *et al*. Study on the Relationship between Th17 Cells and Unexplained Recurrent Spontaneous Abortion. Am J Reprod Immunol 2011;65:503-11.

[51] Aluvihare VR, Betz AG. The Role of Regulatory T Cells in Materno-Fetal Tolerance. In: Immunology of Pregnancy.

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

Berlin, Germany: Springer; 2006. p. 171-8.

[52] Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and Immune Tolerance in Pregnancy: A New Target for Infertility Treatment? Hum Reprod Update 2009;15:517-35.

[53] Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, *et al*. Reciprocal Developmental Pathways for the Generation of Pathogenic Effector TH17 and Regulatory T Cells. Nature 2006;441:235-8.

[54] Yang H, Qiu L, Chen G, Ye Z, Lü C, Lin Q. Proportional Change of CD4+ CD25+ Regulatory T Cells in Decidua and Peripheral Blood in Unexplained Recurrent Spontaneous Abortion Patients. Fertil Steril 2008;89:656-61.

[55] Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal Human Pregnancy is Associated with an Elevation in the Immune Suppressive CD25+ CD4+ Regulatory T-cell Subset. Immunology 2004;112:38-43.

[56] Wang WJ, Hao CF, Yin GJ, Bao SH, Qiu LH, Lin QD. Increased Prevalence of T Helper 17 (Th17) Cells in Peripheral Blood and Decidua in Unexplained Recurrent Spontaneous Abortion Patients. J Reprod Immunol 2010;84:164-70.

[57] Lee S, Kim J, Hur S, Kim C, Na B, Lee M, *et al*. An Imbalance in Interleukin-17-producing T and Foxp3+ Regulatory T Cells in Women with Idiopathic Recurrent Pregnancy Loss. Hum Reprod 2011;26:2964-71.

[58] Wang WJ, Hao CF, Qu QL, Wang X, Qiu LH, Lin QD. The Deregulation of Regulatory T Cells on Interleukin-17-producing T Helper Cells in Patients with Unexplained Early Recurrent Miscarriage. Hum Reprod 2010;25:2591-6.

[59] Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and Peripheral Blood CD4+ CD25+ Regulatory T Cells in Early Pregnancy Subjects and Spontaneous Abortion Cases. Mol Hum Reprod 2004;10:347-53.

[60] Mei S, Tan J, Chen H, Chen Y, Zhang J. Changes of CD4+ CD25 High Regulatory T Cells and FOXP3 Expression in Unexplained Recurrent Spontaneous Abortion Patients. Fertil Steril 2010;94:2244-7.

[61] Wu L, Luo LH, Zhang YX, Li Q, Xu B, Zhou GX, *et al*. Alteration of Th17 and Treg Cells in Patients with Unexplained Recurrent Spontaneous Abortion before and after Lymphocyte Immunization Therapy. Reprod Biol Endocrinol 2014;12:74.

[62] Jasper MJ, Tremellen KP, Robertson SA. Primary Unexplained Infertility is Associated with Reduced Expression of the T-regulatory Cell Transcription Factor Foxp3 in Endometrial Tissue. Mol Hum Reprod 2006;12:301-8.

[63] Sereshki N, Gharagozloo M, Ostadi V, Ghahiri A, Roghaei MA, Mehrabian F, *et al*. Variations in T-helper 17 and Regulatory T Cells during the Menstrual Cycle in Peripheral Blood of Women with Recurrent Spontaneous Abortion. Int J Fertil Steril 2014;8:59.

[64] Hall OJ, Klein SL. Progesterone-based Compounds Affect Immune Responses and Susceptibility to Infections at Diverse Mucosal Sites. Mucosal immunol 2017;10:1097-107.

[65] Rodríguez A, Rodríguez M, Córdoba JJ, Andrade MJ. Design of Primers and Probes for Quantitative Real-time PCR Methods, PCR Primer Design. Berlin, Germany: Springer; 2015. p. 31-56.

[66] Broeders S, Huber I, Grohmann L, Berben G, Taverniers I, Mazzara M, *et al*. Guidelines for Validation of Qualitative Real-time PCR Methods. Trends Food Sci Technol 2014;37:115-26.

[67] Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, *et al*. Evidence for a Selective Migration of Fetus-specific CD4+ CD25bright Regulatory T Cells from the Peripheral Blood to the Decidua in Human Pregnancy. J Immunol 2008;180:5737-45.

[68] Mumford SL, Garbose RA, Kim K, Kissell K, Kuhr DL, Omosigho UR, *et al*. Association of Preconception Serum 25-hydroxyvitamin D Concentrations with Livebirth and Pregnancy Loss: A Prospective Cohort Study. Lancet Diabetes Endocrinol 2018;6:725-32.

[69] Wu M, Liu P, Cheng L. Galectin-1 Reduction and Changes in T Regulatory Cells May Play Crucial Roles in Patients with Unexplained Recurrent Spontaneous Abortion. Int J Clin Exp Pathol 2015;8:1973.

[70] Hou W, Yan X, Bai C, Zhang X, Hui L, Yu X. Decreased Serum Vitamin D Levels in Early Spontaneous Pregnancy Loss. Eur J Clin Nutr 2016;70:1004.

[71] Wang J, Yang J, Yan Y, Zhu Z, Mu Y, Wang X, *et al*. Effect of Adoptive Transfer of CD4+ CD25+ Foxp3+ Treg Induced by Trichostatin A on the Prevention of Spontaneous Abortion. J Reprod Immunol 2019;131:30-5.

[72] Elham A, Rahim R, Nafiseh S, Vicki C, Maryam R, Amirhossein S, *et al*. Evaluation of the Effects of 1,25 Vitamin D3 on Regulatory T Cells and T Helper 17 Cells in Vitamin D-deficient Women with Unexplained Recurrent Pregnancy Loss. Curr Mol Pharmacol 2020;13:1.

[73] Arruvito L, Sotelo AI, Billordo A, Fainboim L. A Physiological Role for Inducible FOXP3+ TREG Cells: Lessons from Women with Reproductive Failure. Clin Immunol 2010;136:432-41.

[74] Rudensky AY. Regulatory T Cells and Foxp3. Immunol Rev 2011;241:260-8.

[75] Rossetti M, Spreafico R, Saidin S, Chua C, Moshref M, Leong JY, *et al*. *Ex Vivo*-Expanded but Not *in Vitro*-induced Human Regulatory T Cells are Candidates for Cell Therapy in Autoimmune Diseases Thanks to Stable Demethylation of the FOXP3 Regulatory T Cell-specific Demethylated Region. J Immunol 2015;194:113-24.

[76] Saxena D, Misra M, Parveen F, Phadke S, Agrawal S. The Transcription Factor Forkhead Box P3 Gene Variants Affect Idiopathic Recurrent Pregnancy Loss. Placenta 2015;36:226-31.

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001

[77] Zidan HE, Abdul-Maksoud RS, Mowafy HE, Elsayed WS. The Association of IL-33 and Foxp3 Gene Polymorphisms with Recurrent Pregnancy Loss in Egyptian Women. Cytokine 2018;108:115-9.

[78] Wu Z, You Z, Zhang C, Li Z, Su X, Zhang X, *et al*. Association between Functional Polymorphisms of Foxp3 Gene and the Occurrence of Unexplained Recurrent Spontaneous Abortion in a Chinese Han Population. Clin Dev Immunol 2012;2012:896458.

[79] Qiu T, Teng Y, Wang Y, Xu L. Adoptive Transfer of Transforming Growth Factor-β1-induced CD4+ CD25+ Regulatory T Cells Prevents Immune Response-Mediated Spontaneous Abortion. Reprod Fertil Dev 2016;28:1788-97.

[80] Sanjabi S, Oh SA, Li MO. Regulation of the Immune Response by TGF-β: From Conception to Autoimmunity and Infection. Cold Spring Harb Perspect Biol 2017;9:a022236.

[81] Zhang S. The Role of Transforming Growth Factor β in T Helper 17 Differentiation. Immunology 2018;155:24-35.

[82] Ogasawara MS, Aoki K, Aoyama T, Katano K, Iinuma Y, Ozaki Y, *et al*. Elevation of Transforming Growth Factor-β1 is Associated with Recurrent Miscarriage. J Clin Immunol 2000;20:453-7.

[83] Lea RG, Underwood J, Flanders KC, Hirte H, Banwatt D, Finotto S, *et al*. A Subset of Patients With Recurrent Spontaneous Abortion Is Deficient in Transforming Growth Factor β-2-Producing "Suppressor Cells" in Uterine Tissue Near the Placental Attachment Site. Am J Reprod Immunol 1995;34:52-64.

[84] Co EC, Gormley M, Kapidzic M, Rosen DB, Scott MA, Stolp HA, *et al*. Maternal Decidual Macrophages Inhibit NK Cell Killing of Invasive Cytotrophoblasts during Human Pregnancy. Biol Reprod 2013;88:155.

[85] Abdollahi E, Tavasolian F, Ghasemi N, Mirghanizadeh SA, Azizi M, Ghoryani M, *et al*. Association between Lower Frequency of R381Q Variant (rs11209026) in IL-23 Receptor Gene and Increased Risk of Recurrent Spontaneous Abortion (RSA). J Immunotoxicol 2015;12:317-21.

[86] Prots I, Skapenko A, Wendler J, Mattyasovszky S, Yoné CL, Spriewald B, *et al*. Association of the IL4R Single-nucleotide Polymorphism I50V with Rapidly Erosive Rheumatoid Arthritis. Arthritis Rheum 2006;54:1491-500.

[87] Nakashima A, Shima T, Inada K, Ito M, Saito S. The Balance of the Immune System between T Cells and NK Cells in Miscarriage. Am J Reprod Immunol 2012;67:304-10.

[88] Mandal M, Marzouk AC, Donnelly R, Ponzio NM. Maternal Immune Stimulation during Pregnancy Affects Adaptive Immunity in Offspring to Promote Development of TH17 Cells. Brain Behav Immunity 2011;25:863-71.

[89] Abdollahi E, Rezaee SA, Saghafi N, Rastin M, Clifton V, Sahebkar A, *et al*. Evaluation of the Effects of 1, 25 Vitamin D3 on Regulatory T Cells and T Helper 17 Cells in Vitamin D-deficient Women with Unexplained Recurrent Pregnancy Loss. Curr Mol Pharmacol 2020;13:306-17.

[90] Abdollahi E, Saghafi N, Rezaee SA, Rastin M, Jarahi L, Clifton V, *et al*. Evaluation of 1, 25 (OH) 2D3 Effects on FOXP3, ROR-γt, GITR, and CTLA-4 Gene Expression in PBMCs of Vitamin D-Deficient Women with Unexplained Recurrent Pregnancy Loss. Iran Biomed J 2020;24:290.

[91] Parva NR, Tadepalli S, Singh P, Qian A, Joshi R, Kandala H, *et al*. Prevalence of Vitamin D Deficiency and Associated Risk Factors in the US Population (2011-2012). Cureus 2018;10:e2741.

[92] Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: Modulator of the Immune System. Curr Opin Pharmacol 2010;10:482-96.

[93] Marchesini G, Marchignoli F, Petta S. Evidence-Based Medicine and the Problem of Healthy Volunteers. Ann Hepatol 2017;16:832-4.

[94] Ota K, Dambaeva S, Han AR, Beaman K, Gilman-Sachs A, Kwak-Kim J. Vitamin D Deficiency May be a Risk Factor for Recurrent Pregnancy Losses by Increasing Cellular Immunity and Autoimmunity. Hum Reprod 2013;29:208-19.

DOI: http://dx.doi.org/10.18053/jctres.08.202203.001