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

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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
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 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 placentation development [4,31]. TGF-β has been shown to maintain peripheral natural iTregs 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-17A+) is characterized by the expression of IL-17A, IL-17F, 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%).

\[
n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 (S_1^2 + S_2^2)}{(X_1 - X_2)^2}
\]

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.
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                                         |
| Body mass index (BMI) | 23.3±5.9                                       |
| Mean number of miscarriages | 4 (range=3–7 miscarriages) |
| Mean time of miscarriage (week of gestation) | 5.5                                       |
| Mean 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          | Negative                                         |

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/µL) using control cDNA (25 ng/µL, 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
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-\( \beta \) gene expression in patients with URPL and the controls

TGF-\( \beta \) 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).

3.3. TGF-\( \beta \) cervicovaginal fluid levels in patients with URPL and the controls

The levels of cervicovaginal fluid TGF-\( \beta \) 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-\( \beta \) 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

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

| Targeted gene | Sequence (5' to 3') | Product size (bp) |
|---------------|---------------------|-------------------|
| FOXP3 | CACCTGGAAGAACGCCATCC Forward | 94 |
| | CTCATCCACCGTCCACACAG Reverse | |
| | CCCCTCTGCTCTCCACCGCACA Probe | |
| | TCACCTGCTCCACGGCCTTGCTCT Probe | |
| IL-17A | GGAATCTCCACCGCAATGAGG Forward | 130 |
| | TGCTGGATGGGACAGAGTTC Reverse | |
| | TGTTAGTCCACGTTCCTCACAGCG Probe | |
| TGF-\( \beta \) | CCAGAAATACAGCAACAATTCCTG Forward | 136 |
| | GCCCTCAATTTCCCCCTCACACCGA Reverse | |
| | CTAACCACTGCGCACAACACCTCGG Probe | |
| 18s rRNA | GTTGATTAAGTCCCTCCTCTTGG Forward | 152 |
| | TCCAGGGGCTTC ACTAAACC Reverse | |
| | ACACACGCCGCGT GCTACACCGG Probe | |

RT-PCR: Reverse transcription polymerase chain reaction; FOXP3: Forkhead box P3; IL-10: Interleukin 10; TGF-\( \beta \): 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

### 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 \( P < 0.05 \).
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 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. *Represents \( P < 0.05 \).

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. *Represents \( P < 0.05 \).

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 \( P < 0.01 \).
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 URPL 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
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.

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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.

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