

# Distinct pattern of Th17/Treg cells in pregnant women with a history of unexplained recurrent spontaneous abortion

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## Summary

The aim of the current study was to determine the pattern of immune cells and related functional molecules in peripheral blood and at the maternal-fetal interface in women with unexplained recurrent spontaneous abortion (URSA). In part I, 155 women were included and divided into four groups: non-pregnant controls with no history of URSA (NPCs), pregnant controls with no history of URSA (PCs), non-pregnant women with a history of URSA (NPU), and pregnant women with a history of URSA (PU). Venous blood samples were collected and analyzed. In part II, 35 subjects with URSA and 40 subjects in the early stage of normal pregnancy who chose to undergo an abortion were recruited. Samples of the decidua were collected, and the proportion of immune cells and the expression of related molecules were evaluated. Peripheral regulatory T cells (Treg cells) increased in PCs compared to NPCs, but in women with URSA the flux of Treg cells disappeared when pregnancy occurred. Levels of interleukin-10 (IL-10), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and IL-17 and the ratio of Th17/Treg cells in peripheral blood remained stable among the four groups. At the maternal-fetal interface, the percentage of Treg cells, the level of CTLA-4 of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells and CD4<sup>+</sup>Foxp3<sup>+</sup> cells were significantly lower in women with URSA compared to controls, respectively. Levels of transforming growth factor-β1 (TGF-β1) mRNA and protein in the decidua significantly decreased in URSA while levels of IL-6 and tumor necrosis factor-α (TNF-α) and the Th17/Treg ratio significantly increased. In conclusion, peripheral Treg cells did not increase in pregnant women with URSA. The decrease in Treg cells and levels of CTLA-4 and TGF-β1 and as well as the increase in levels of IL-6 and TNF-α, and the Th17/Treg ratio at the maternal-fetal interface might contribute to inappropriate maternal-fetal immune tolerance in URSA.

**Keywords:** Treg cells, Th17 cells, unexplained recurrent spontaneous abortion, cytokine, maternal-fetal tolerance

Released online in J-STAGE as advance publication April 15, 2018.

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## 1. Introduction

Recurrent spontaneous abortion (RSA) refers to two or more consecutive pregnancy losses before 20 weeks of gestation. RSA affects about 1% of all women and results in physical and psychological distress. The common causes of RSA include parental or embryonic karyotype anomalies, uterine anatomic abnormalities, infection, endocrine disorders, and antiphospholipid syndrome (1). However, nearly 50% of cases remain unexplained (2). Maternal-fetal immune abnormalities

might be one cause of unexplained recurrent spontaneous abortion (URSA) (3).

The human fetus is a semi-allograft and antigenically foreign to the mother. Maternal-fetal immune tolerance plays a significant role in establishing and maintaining a successful pregnancy. CD4<sup>+</sup> T cells, also known as Th cells, are crucial to this process. CD4<sup>+</sup> T cells can be classified into Th1 cells, Th2 cells, regulatory T cells (Treg cells), and Th17 cells (4). Previously, a successful pregnancy was believed to be associated with Th2-dominant immunity, and an imbalance in Th1/Th2 cells was believed to result in spontaneous abortion (5). However, Th2-dominance was later found in URSA (6). Therefore, the Th1/Th2 paradigm was no longer sufficient to explain the maternal-fetal tolerance, and a new paradigm of Th1/Th2/Th17/Treg cells has been developed.

Treg and Th17 cells share the same origin and have the opposite effects on inflammation. Treg cells express surface markers such as CD25 (7), CD127 (low expression, human only) (8), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (9), and glucocorticoid induced tumor necrosis factor receptor-related protein (GITR) (10). Forkhead box protein 3 (Foxp3) serves as a unique intracellular transcription factor for the development of Treg cells (11). The main cytokines secreted by Treg cells include interleukin-10 (IL-10) and transforming growth factor  $\beta$  (TGF- $\beta$ ). Treg cells exhibit anti-inflammatory and immune-suppressive action mainly through cytokines and contact-dependent suppression (12). Th17 cells play an important role in autoimmunity and the protective response against extracellular fungi and bacteria by releasing pro-inflammatory cytokines such as IL17, IL-6, IL-22, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (13). Th17 cells are regulated by transcription factor retinoic acid receptor-related orphan receptors (ROR) (14).

The essential role of Treg cells in growth and development of the fetus in humans and mice has been widely reported (15). The function of Th17 cells in maternal-fetal tolerance has not been fully elucidated. Changes in the proportion of Th17 cells in women in the early stage of normal pregnancy compared to the proportion in non-pregnant women are not consistent (16). Recent findings indicated that Th17 cells might participate in pregnancy-related complications including spontaneous abortions and pre-eclampsia (17). The Th17/Treg ratio shifts in favor of Treg cells in a health pregnancy, and a decrease in Treg cells or an increase in Th17 cells is detrimental to normal pregnancy (18). However, the roles of Th17 cells and the balance in Th17/Treg cells in the early stage of pregnancy with URSA are still unclear.

The current study focused on the pattern of Th17/Treg cells and the expression of related molecules in pregnant women with a history of URSA in peripheral blood as well as at the maternal-fetal interface.

## 2. Materials and Methods

### 2.1. Study population

Study subjects ages 18 to 40 years were recruited from the Obstetrics and Gynecology Hospital of Fudan University in China between August 2017 and March 2018. To investigate the peripheral Th17/Treg balance in part I, a total of 155 women were divided into four groups and venous blood samples were collected: 40 non-pregnant controls with no history of URSA (NPCs), 40 pregnant controls with no history of URSA (PCs), 38 non-pregnant women with a history of URSA (NPU), 37 pregnant women with a history of URSA (PU). To study the immune tolerance at the maternal-fetal interface in part II, 35 subjects with URSA and 40 subjects in the early stage of normal pregnancy who chose to undergo an abortion were recruited, and samples of the aborted tissue were collected. Women with a history of two or more consecutive spontaneous abortions due to unexplained causes (excluding parental or embryonic karyotype anomalies, uterine anatomic abnormalities, an infection, endocrine disorders, and antiphospholipid syndrome) before 20 weeks of gestation were diagnosed as having URSA. Women with at least one successful pregnancy or no history of spontaneous abortions served as controls. In pregnant women, the gestational age was from 45 to 90 days when samples were collected. None of the subjects had a history of smoking, alcohol addiction, liver or renal diseases, or vaccination in three months before recruitment. This study was approved by the ethics committee of the Obstetrics and Gynecology Hospital of Fudan University. All subjects provided written informed consent.

### 2.2. Blood and tissue samples

Peripheral blood mononuclear cells (PBMCs) were isolated from 5 mL of heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (2,500 rpm at room temperature for 20 minutes). Cells were collected at the interface and washed with phosphate buffered saline (PBS). The decidua was aseptically collected within 5 minutes after artificial abortion and washed with sterile saline. One sample was stored at -80°C for a real-time quantitative polymerase chain reaction (RT-PCR) and an enzyme-linked immunosorbent assay (ELISA), and another sample was placed in DMEM/F12 culture for cell separation. In brief, tissue was digested with collagenase and DNase at 37°C for 30 minutes in a shaking water bath. The released cells were then separated by filtering through a nylon mesh, and the cells were centrifuged at 1,500 rpm for 8 minutes at room temperature. Percoll was added to the mixture, which was centrifuged at 2,500 rpm for 30 minutes. The cells were stratified into a dead cell layer,

a decidual stromal cell layer, a decidual immune cell layer, and an erythrocyte layer. The decidual immune cell layer was preserved, washed with PBS, and then centrifuged at 1,500 rpm (4°C) for 8 minutes to obtain a precipitate.

### 2.3. Flow cytometry analysis

Collected cells were divided into stimulated and unstimulated samples. Surface chemokine data were obtained from the unstimulated samples. CD4, CD25, CD127, and CTLA-4/PEcy7 were used to perform surface staining for 30 minutes in the dark. To stain the intracellular molecule Foxp3, cells were permeabilized with a permeabilization/fixation buffer and stained with antihuman Foxp3-PE antibody followed by surface staining. The stimulated samples were used to stain the intracellular molecules IL-17A and IL-10. Cells were permeabilized with a permeabilization/fixation buffer and stained with antihuman IL17A-Alexa 647 and anti-human IL-10-Alexa 647 antibody (Biolegend), followed by surface staining. CyAn ADP flow cytometer (Beckman Coulter) was used to analyze the samples. Postacquisition FACS data were analyzed using the program Flowjo (Ashland, OR, USA).

### 2.4. RT-PCR analysis

Total RNA was isolated from decidual tissue. Briefly, 1 mL of Trizol per 100 mg of tissue was added to tissue and the two were mixed thoroughly on ice. After incubation at room temperature for 10 minutes, 200 µL of chloroform was added and the mixture was centrifuged at 12,000 rpm for 15 minutes. The upper aqueous phase containing RNA was transferred to another 1.5-mL sterile RNase-free EP tube and precipitated by adding an equal volume of 100% isopropanol. The RNA was concentrated by centrifugation at 12,000 rpm for 10 minutes (4°C) and washed with 75% ethanol twice. The resulting RNA pellet was then air-dried and dissolved in 30 µL RNase-free water. A reverse transcription reaction was performed at a final volume of 20 µL containing 500 ng of the RNA sample and 2 µL of 5×PrimeScript RT Master Mix (Perfect Real Time). Afterwards, quantitative real-time PCR was performed on a 20-µL mixture of 2 µL of reverse transcription product, 1.6 µL of a forward/reverse primer mixture for a final concentration of 0.4 µM, 10 µL of 2×SYBR Premix Ex TaqII (Tli RNaseH Plus), and DNase-free water added to reach the final volume. Reactions were performed using the following protocol: 95°C for 30 seconds and cycles of 95°C for 5 seconds and 60°C for 30 seconds for 40 cycles, followed by a dissociation stage. The primers used are listed in Table S1 (<http://www.biosciencetrends.com/action/getSupplementalData.php?ID=23>). Gene expression was normalized to the level of the house-keeping gene β-actin.

### 2.5. ELISA testing

Concentrations of IL-6, IL-10, IL-17, IL-23, TGF-β1, and TNF-α were measured with ELISA according to the manufacturer's instructions (human IL-6 ELISA kit, human IL-10 ELISA kit, human IL-17 ELISA kit, human IL-23 ELISA kit, human TGF-β1 ELISA kit, and human TNF-α ELISA kit; Xitang Biotech Co., Ltd., Shanghai, China). Optical density values were read at 450 nm using a microplate reader (Thermo Fisher Scientific, USA).

### 2.6. Statistical analysis

The Statistical Package for Social Sciences (SPSS) 17.0 was used for statistical analysis. Data are presented as the mean ± SEM. Before analysis, the data were checked for normality. In part I, data differences were analyzed using one-way ANOVA and a Kruskal-Wallis test. In part II, data with a normal distribution were statistically analyzed using the Student's *t*-test, and non-normal data were statistically assessed with the Wilcoxon signed-rank test.  $P < 0.05$  was considered statistically significant.

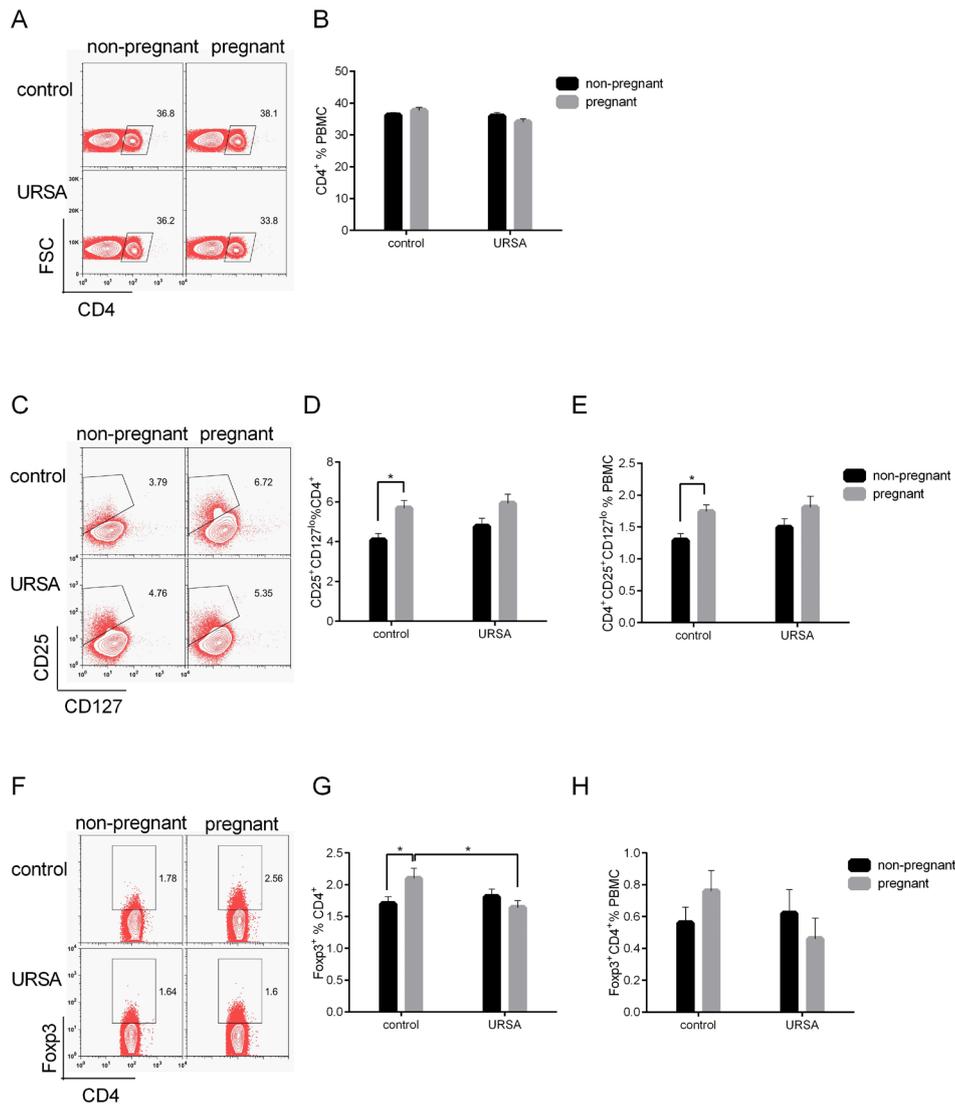
## 3. Results

### 3.1. Clinical characteristics

To investigate the peripheral Th17/Treg balance in part I, a total of 155 women were recruited. Seventy-five subjects had a history of URSA with at least two consecutive first trimester abortions (range: 2-5). There were no significant differences among the four groups in terms of age, body mass index (BMI), and gestational age (Table S2, <http://www.biosciencetrends.com/action/getSupplementalData.php?ID=23>). To study the immune tolerance at the maternal-fetal interface in part II, 75 subjects were recruited. There were no significant differences between the two groups in terms of age, BMI, gestational age, and gravidity (Table S3, <http://www.biosciencetrends.com/action/getSupplementalData.php?ID=23>).

### 3.2. Peripheral Treg cells in pregnant women with URSA

Results indicated that there was no significant difference in the percentage of CD4<sup>+</sup> T cells in PBMCs among the four groups ( $F = 2.160$ ,  $P = 0.095$ ) (Figure 1 A, B). The percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells among CD4<sup>+</sup> T cells was significantly higher in PCs than that in NPCs ( $t = 3.165$ ,  $P = 0.002$ ) (Figure 1 C, D). However, in women with URSA, there was no elevation of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells among CD4<sup>+</sup> T cells in PUs compared to NPU ( $t = 1.882$ ,  $P = 0.064$ ) (Figure 1 C, D). The proportion of



**Figure 1. Pattern of peripheral Treg cells in URSA.** (A, B) There was no significant difference in the percentage of CD4<sup>+</sup> T cells in PBMCs among the four groups. (C, D, E) The percentage of CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells in CD4<sup>+</sup> T cells increased in pregnant controls (PCs) compared to that in non-pregnant controls (NPCs). CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells did not increase in pregnant women with a history of URSA (PUs) compared to non-pregnant women with a history of URSA (NPU). Similar results were obtained for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells in PBMCs. (F, G, H) The proportion of Foxp3<sup>+</sup> Treg cells in CD4<sup>+</sup> T cells was higher in PCs compared to NPCs and lower in PUs than in PCs while remaining stable in PUs compared to NPU. The proportion of Foxp3<sup>+</sup> Treg cells in PBMCs did not differ significantly among the four groups. (The FACS picture represents an individual sample. Data represent the mean ± SEM. \*p < 0.05.

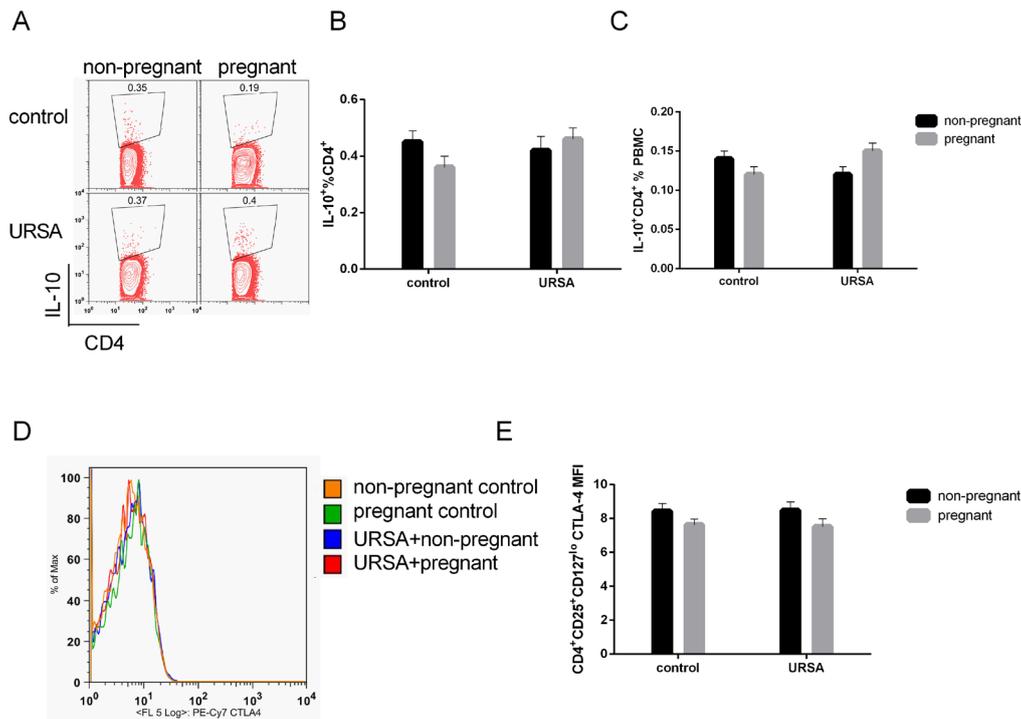
CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells in PBMCs was higher in PCs than that in NPCs (t = 2.905, P = 0.005) whereas there was no significant difference between that in PUs and that in NPUs (t = 1.465, P = 0.147) (Figure 1 C, D, E).

Results for Foxp3<sup>+</sup> Treg cells indicated that the percentage of Treg cells among CD4<sup>+</sup> T cells increased in PCs compared to that in NPCs (t = 2.070, P = 0.042) while remaining stable between that in PUs and NPUs (t = 1.015, P = 0.313) (Figure 1 F, G). The proportion of Foxp3<sup>+</sup> Treg cells in CD4<sup>+</sup> T cells was significantly lower in PUs than that in PCs (t = 2.430, P = 0.018) (Figure 1 F, G). The proportion of Foxp3<sup>+</sup> Treg cells in PBMCs did not differ significantly among the four groups (Figure 1 F, G, H).

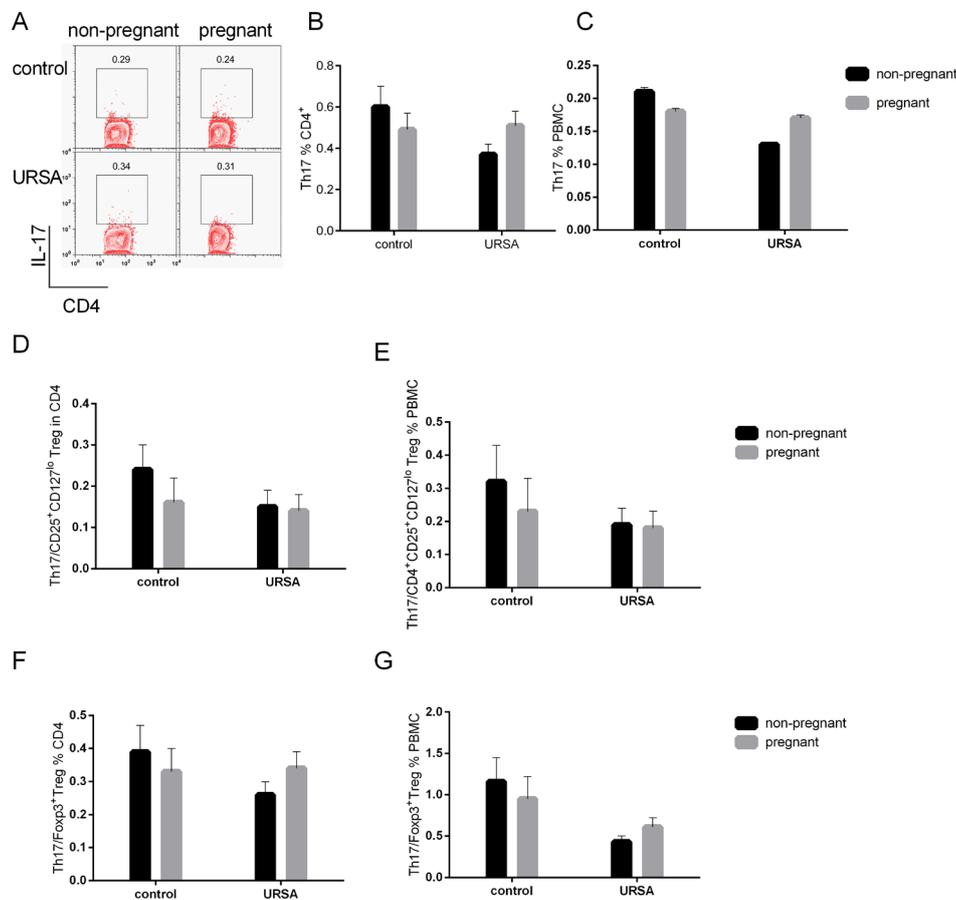
### 3.3. The expression of IL-10 and CTLA-4 in peripheral blood of women with a history of URSA

The expression of IL-10 and CTLA-4 was studied as a possible pathway for the action of Treg cells. Results indicated that there was no significant difference in the expression of IL-10 by CD4<sup>+</sup> T cells (F = 0.975, P = 0.406) (Figure 2 A, B) or IL-10<sup>+</sup>CD4<sup>+</sup> T cells in PBMCs among the four groups (F = 1.043, P = 0.376) (Figure 2 C). In addition, there was no significant difference in the expression of CTLA-4 on CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells among the groups (F = 1.301, P = 0.276) (Figure 2 D, E).

### 3.4. The ratio of peripheral Th17/Treg cells in women with a history of URSA



**Figure 2. Expression of IL-10 and CTLA-4 in peripheral blood of women with URSA.** (A, B, C) There was no significant difference in the expression of IL-10 by CD4<sup>+</sup> T cells or PBMCs among the four groups. (D, E) There was no significant difference in the CTLA-4 mean fluorescence intensity (MFI) of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells among the groups. (The FACS picture represents an individual sample. Data represent the mean  $\pm$  SEM. \* $p < 0.05$ .)



**Figure 3. The ratio of peripheral Th17/Treg in URSA.** (A, B, C) There was no significant difference in the expression of IL-17 by CD4<sup>+</sup> T cells or PBMCs among the four groups. (D, E, F, G) There was no significant difference in the ratio of Th17/Treg cells among CD4<sup>+</sup> T cells or PBMCs among the groups. (The FACS picture represents an individual sample. Data represent the Mean  $\pm$  SEM. \* $p < 0.05$ .)

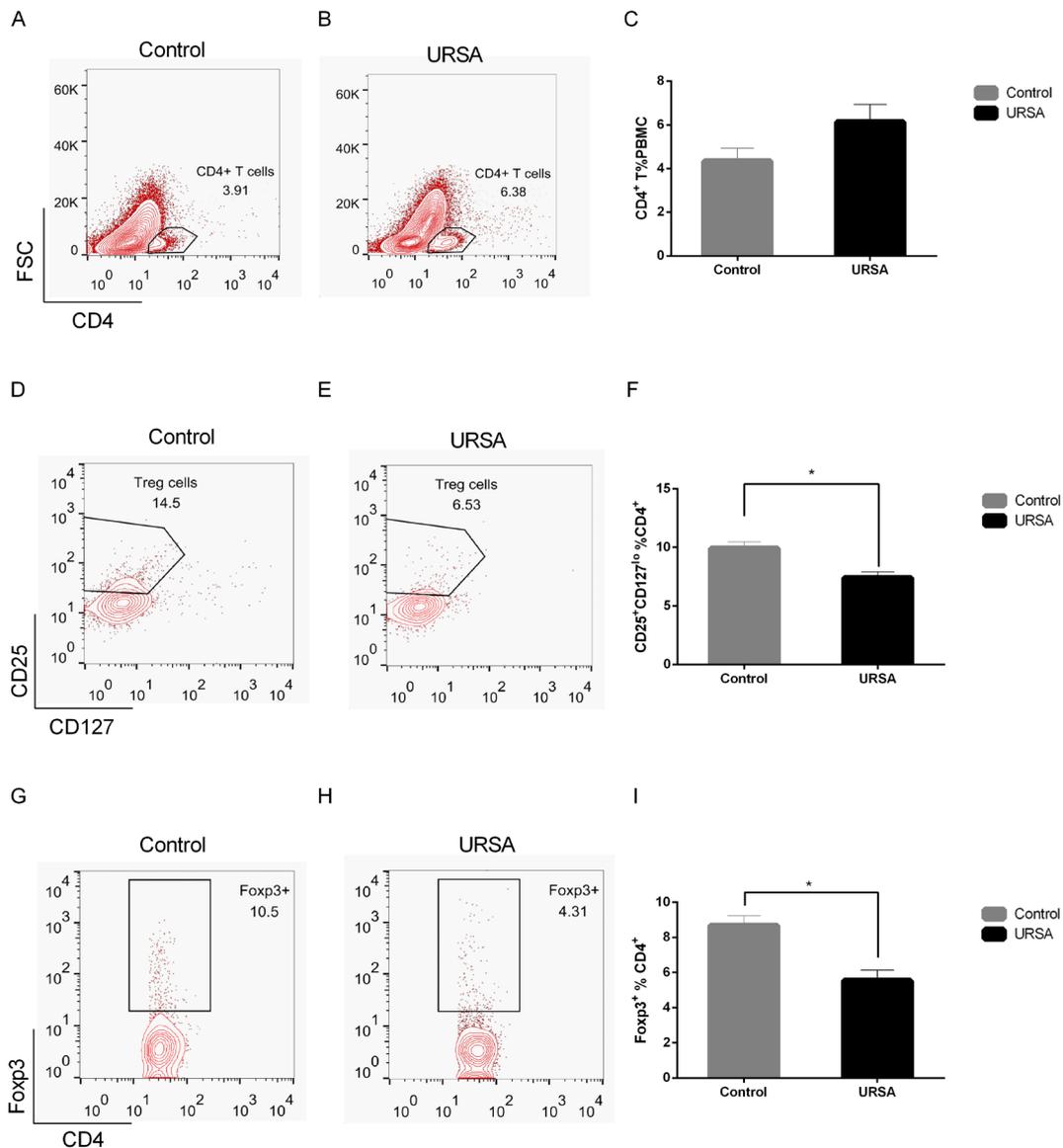
IL-17 is a characteristic cytokine of Th17 cells. The current study found that IL-17<sup>+</sup> cells among CD4<sup>+</sup> T cells or PBMCs remained stable among the four groups ( $F = 1.386, P = 0.250$ ;  $F = 1.099, P = 0.352$ ) (Figure 3 A, B, C). The ratio of Th17/Treg cells was examined, and results indicated that there was no statistical difference in either CD4<sup>+</sup> T cells (Th17/CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg;  $Z = 3.305, P = 0.347$ ; Th17/Foxp3<sup>+</sup> Treg;  $Z = 4.919, P = 0.178$ ) or PBMCs (Th17/CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg;  $Z = 2.610, P = 0.456$ ; Th17/Foxp3<sup>+</sup> Treg;  $Z = 2.732, P = 0.435$ ) among the four groups (Figure 3 D, E, F, G).

3.5. Treg and Th17 cells and related molecules at the maternal-fetal interface in URSA

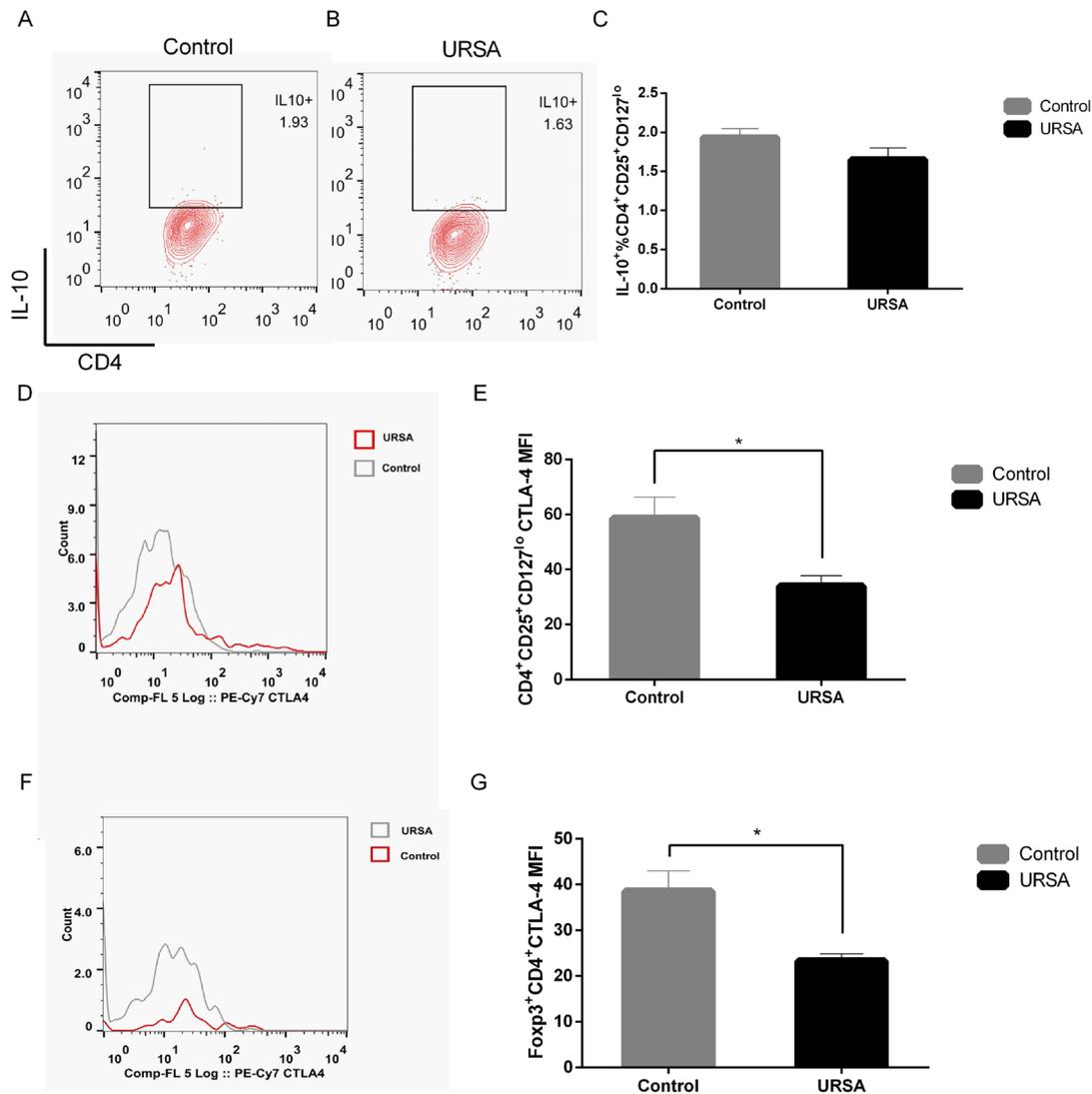
Flow cytometry was also used to detect the proportion of

Treg cells and Th17 cells and the expression of related molecules in decidual immune cells. Results indicated that the proportion of CD4<sup>+</sup> T cells in decidual immune cells did not differ significantly between women with URSA and women with a normal pregnancy ( $P > 0.05$ ) (Figure 4 A, B, C). The percentage of CD25<sup>+</sup>CD127<sup>lo</sup> cells and Foxp3<sup>+</sup> cells in CD4<sup>+</sup> T cells was significantly lower in women with URSA than that in women in the early stage of normal pregnancy ( $P < 0.05$ ) (Figure 4 D, E, F, G, H, I).

IL-10 expression by CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> T cells did not differ significantly between women with URSA and women with a normal pregnancy ( $P > 0.05$ ) (Figure 5 A, B, C). However, women with URSA had a significantly lower expression of CTLA-4 MFI on CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells or CD4<sup>+</sup>Foxp3<sup>+</sup> cells



**Figure 4. Proportion of CD4<sup>+</sup> T cells and Treg cells in decidual immune cells.** (A, B, C) There was no significant difference in the proportion of CD4<sup>+</sup> T cells in the decidual immune cells between women with URSA and women with a normal pregnancy. (D, E, F) Women with URSA had a significantly lower percentage of CD25<sup>+</sup>CD127<sup>lo</sup>/CD4<sup>+</sup> T cells than their counterparts. (G, H, I) Women with URSA had a significantly lower percentage of Foxp3<sup>+</sup>/CD4<sup>+</sup> T cells. (The FACS picture represents an individual sample. Data represent the Mean ± SEM. \* $p < 0.05$ .)



**Figure 5. Expression of related molecules in decidual immune cells.** (A, B, C) There was no significant difference in the IL-10 expression of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> T cells between women with URSA and women with a normal pregnancy. (D, E) Women with URSA had a significantly lower MFI of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> CTLA-4 expression than other groups. (F, G) Women with URSA had a significantly lower MFI of Fopx3<sup>+</sup> CTLA-4 expression. (The FACS picture represents an individual sample. Data represent the Mean  $\pm$  SEM. \* $p < 0.05$ .)

compared to the control group respectively ( $P < 0.05$ ) (Figure 5 D, E, F, G).

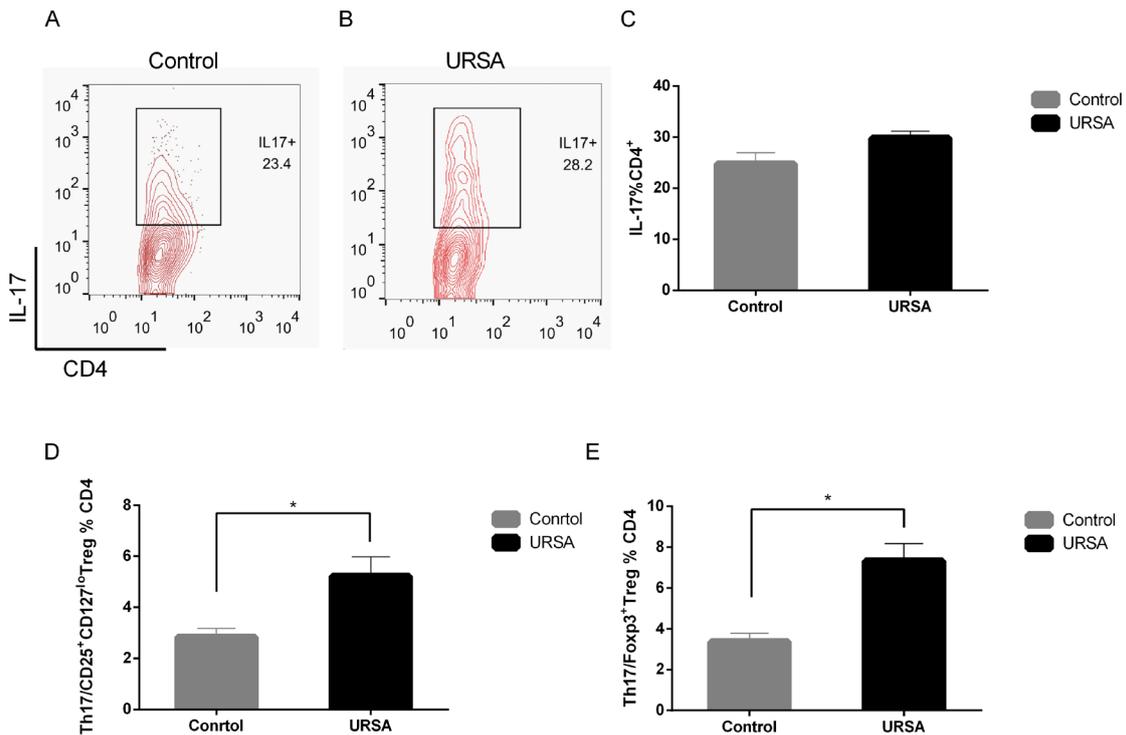
The percentage of IL-17<sup>+</sup> cells among CD4<sup>+</sup> T cells was slightly higher in women with URSA than that in women with a normal pregnancy, but the difference was not statistically significant ( $P > 0.05$ ) (Figure 6 A, B, C). The ratio of Th17/CD25<sup>+</sup>CD127<sup>lo</sup> Treg and Th17/Fopx3<sup>+</sup> Treg in women with URSA was significantly higher than that in women with a normal pregnancy ( $P < 0.05$ ) (Figure 6 D, E).

### 3.6. Levels of mRNA and protein of related molecules at the maternal-fetal interface in URSA

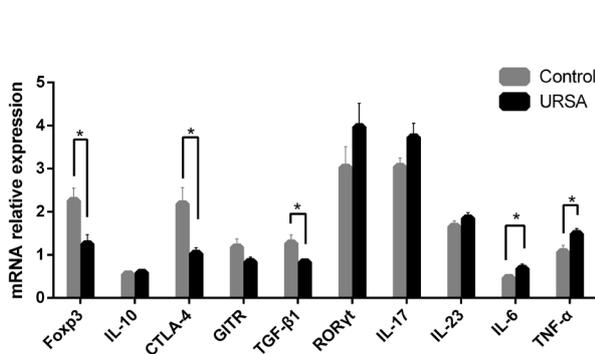
RT-PCR was used to detect the level of transcription of related molecules at the maternal-fetal interface in

women with URSA. Results indicated that levels of IL-6 and TNF- $\alpha$  mRNA in decidual tissue from women with URSA were significantly higher than those in women with a normal pregnancy ( $P < 0.05$ ). Levels of Fopx3, CTLA-4, and TGF- $\beta$ 1 mRNA were significantly lower ( $P < 0.05$ ), while levels of IL-10, IL-17, IL-23, GITR, and ROR $\gamma$ t mRNA did not statistically differ between the two groups ( $P > 0.05$ ) (Figure 7).

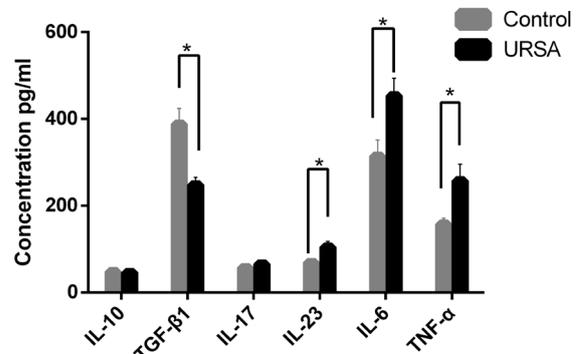
The level of expression of related proteins in decidua was detected with ELISA. The levels of IL-6, IL-23, and TNF- $\alpha$  protein in women with URSA were significantly higher than those in women in the early stage of normal pregnancy ( $P < 0.05$ ) and the level of TGF- $\beta$ 1 protein in women with URSA was significantly lower ( $P < 0.05$ ), while the levels of IL-17 and IL-10 did not statistically differ between the two groups ( $P > 0.05$ ) (Figure 8).



**Figure 6. Proportion of Th17 cells and the ratio of Th17/Treg cells in decidual immune cells.** (A, B, C) There was no significant difference in the percentage of IL-17<sup>+</sup> cells in CD4<sup>+</sup> T cells between women with URSA and women with a normal pregnancy. (D) Women with URSA had a significantly higher proportion of Th17/CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg cells than that in the control group. (E) Women with URSA had a significantly higher ratio of Th17/Foxp3<sup>+</sup> Treg cells. (The FACS picture represents an individual sample. Data represent the Mean ± SEM. \**p* < 0.05).



**Figure 7. Levels of mRNA of related functional molecules at the maternal-fetal interface in URSA.** (Data represent the Mean ± SEM. \**p* < 0.05.)



**Figure 8. Levels of proteins of related functional molecules at the maternal-fetal interface in URSA.** (Data represent the mean ± SEM. \**p* < 0.05.)

**4. Discussion**

The aim of this study was to determine the pattern of Th17/Treg cells and related molecules in early pregnant women with a history of URSA. The immune system finely regulates the immune response in the human body to both fight infection and to protect itself from antigens (19). A successful pregnancy relies on the immune tolerance response, which enables the mother to tolerate the semi-allogeneic fetus. Without a delicate maternal-fetal immune balance, a series of pregnancy-related complications will occur, such as spontaneous abortion, preterm birth, and pre-eclampsia.

Several studies have indicated that a deficiency in Treg cells results in implantation failures and miscarriages in mice (20,21). The abortion rate can be reduced by transforming Treg cells in abortion-prone mice (22,23). In human pregnancy, Treg cells are found both in peripheral blood and deciduae. Somerset *et al.* (24) and Liu *et al.* (18) found that Treg cells in PBMCs significantly increased in the first trimester compared to the cell count in non-pregnant women. Most studies have noted an augmentation of Treg cells during the first and second trimester of pregnancy (25), while diminished levels of Treg cells have been reported in the second trimester of pregnancy (26). In the third trimester, Treg

levels decreased and further declined after labor (27). Studies have also indicated that the levels of Treg cells were significantly higher in normal pregnancy than those in spontaneous abortions (28,29). In assisted reproductive techniques (ART), the levels of Treg cells were higher in women who had a successful pregnancy and live birth than those in women with a failed pregnancy (30,31). The current study also found that peripheral Treg cells increased in PCs compared to NPCs. However, the elevation of Treg cells disappeared when pregnancy occurred in women with a history of URSA. Moreover, the proportion of Treg cells was significantly lower in women with URSA than that in women in the early stage of normal pregnancy both in the peripheral blood and at the maternal-fetal interface. This indicates that the immune response in pregnant women with a history of URSA differs from that in pregnant controls. The distinct pattern of immune response in women with URSA suggests that women with URSA are less likely to develop maternal-fetal immune tolerance during re-pregnancy.

IL-10, which is mainly secreted by Treg cells, is known to be an effective immune-regulating cytokine and an inhibitor of inflammatory cytokine synthesis. CTLA-4, a surface marker of Treg cells, may exhibit regulatory action in a contact-dependent manner. However, the function of IL-10 and CTLA-4 during pregnancy is still debated. Several studies found that IL-10 controlled the inflammatory process in pregnancy and that any change in the level of IL-10 might cause a disturbed pregnancy including URSA (32,33), while another study suggested that a deficiency in IL-10 had no effect on a disturbed pregnancy (34). Treg cells did not provide protection through CTLA-4 in a murine model of fetal tolerance (35,36). In contrast, a human study by Jin *et al.* proposed a role for CTLA-4 in Treg function (37). In the current study, there was no significant change in IL-10 expression either in peripheral blood or at the maternal-fetal interface after pregnancy, regardless of whether the woman had a history of URSA or not. Expression of CTLA-4 at the maternal-fetal interface decreased in women with URSA compared to that in pregnant controls, while peripheral CTLA-4 did not differ significantly among the groups. Further work is required to explore the mechanism of CTLA-4 in URSA.

IL-17, which is mainly secreted by Th17 cells, is a pro-inflammatory cytokine. The role of IL-17 and Th17 cells during pregnancy remains in question. Nakashima *et al.* found that the number of circulating Th17 cells did not change during different stages of pregnancy (non-pregnancy, first trimester, second trimester, or third trimester) (38). Liu *et al.* found that circulating Th17 cells did not differ markedly between women in the early stage of normal pregnancy and non-pregnant women (18). Another study reported that the proportion of Th17 cells in PBMCs was elevated in women in the early stage of normal pregnancy compared to that in

non-pregnant women (16). Lissauer *et al.* found that Th17 cell frequencies were remarkably heterogeneous among subjects, and a cross-sectional analysis revealed no difference in the percentage of Th17 cells in different stages of gestation (39). A longitudinal analysis depicted a 60% decline in Th17 cells between the first and second trimester of pregnancy. Since the uterine cavity is not sterile, the stable level of Th17 cells may also help to fight against extracellular pathogens. The abnormal elevation of IL-17 at the maternal-fetal interface might result in a miscarriage in mice, and a transfer of Treg cells before mating or administration of anti-IL-17 antibody might prevent URSA (40). The level of IL-17 is higher in peripheral blood in women with URSA compared to that in non-pregnant women (41). Treg cells decreased and Th17 cells increased during the window of implantation in women with a history of URSA (42,43). However, Nakashima *et al.* found that the proportion of IL-17 in a missed abortion did not statistically differ from that in normal pregnancy and that IL-17 was significantly higher in an inevitable abortion with active bleeding than that in a pregnancy with a normal decidua (44). Thus, IL-17 might not be responsible for an abortion in the initial stage, but it might be responsible in the later stage when inflammation develops. Thus, the exact role of Th17 cells and IL-17 in maternal-fetal tolerance is still uncertain. The current study found that IL-17<sup>+</sup> CD4<sup>+</sup> T cells were present in peripheral blood in both pregnant and non-pregnant women and that the proportion of those cells did not differ significantly between non-pregnant and pregnant women. At the maternal-fetal interface, there was no significant difference in the proportion of Th17 cells, and there was no significant difference in the level of IL-17 protein between women with URSA and women with a normal pregnancy.

An imbalance in Th17/Treg cells may be involved in autoimmune, inflammatory, and allergic conditions and pregnancy-related complications. The ratio of Th17/Treg cells was found to increase in women with URSA compared to that in healthy controls (45,46). The current results indicated that the ratio of peripheral Th17/Treg remained stable among the four groups while women with URSA had higher Th17/Treg ratio at the maternal-fetal interface than that in women with a normal pregnancy. This finding suggests that a balance in Th17 and Treg cells might be crucial to maternal-fetal immune tolerance.

## 5. Conclusion

The current study determined the pattern of immune cells and related molecules in URSA at the maternal-fetal interface as well as in peripheral blood. Results indicated that peripheral Treg cells did not increase when women with a history of URSA became pregnant, and this pattern was distinct from that in women with no history of URSA, suggesting that an alteration in

Treg cells might be involved in women with a history of URSA becoming pregnant. The decrease in Treg cells, CTLA-4, and TGF- $\beta$ 1 as well as the increase in levels of IL-6 and TNF- $\alpha$  and the Th17/Treg ratio at the maternal-fetal interface might contribute to inappropriate maternal-fetal immune tolerance in URSA. Recognition of the immune cell pattern in women with a history of URSA is important to understanding the complicated mechanism of human reproduction. Two limitations of this study are the small sample size and lack of data on pregnancy outcomes. Further studies are required to elucidate the mechanism of immune tolerance.

### Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no. 31571196 to Ling Wang), the 2015 Program to Guide Medicine ("YixueYindao") of the Shanghai Municipal Science and Technology Commission grant no. 15401932200 to Ling Wang), the FY2008 JSPS Postdoctoral Fellowship for Foreign Researchers (P08471 to Ling Wang), the National Natural Science Foundation of China (grant no. 30801502 to Ling Wang), the Shanghai Pujiang Program (grant no. 11PJ1401900 to Ling Wang), the Shanghai Project for Development of Leading Disciplines-Integrative Medicine (grant no. 20150407) and the Science Foundation for Young Scientists of the Shanghai Municipal Commission of Health and Family Planning (grant no. 20174Y0224 to Na Zhang).

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(Received January 30, 2018; Revised March 20, 2018; Accepted March 22, 2018)