Alteration of Th17 and Foxp3$^+$ regulatory T cells in patients with unexplained recurrent spontaneous abortion before and after the therapy of hCG combined with immunoglobulin

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Abstract. We conducted this study to investigate the effects of the combination therapy of human chorionic gonadotropin (hCG) plus immunoglobulin (IG) on the levels of T helper 17 (Th17) cells and Foxp3$^+$ regulatory T cells (Treg) in patients with unexplained recurrent spontaneous abortion (URSA). Twenty pregnant women with URSA underwent combination therapy of hCG plus IG. Flow cytometry was performed to measure the proportions of Th17 and Treg cells before and after treatment. RT-PCR and ELISA were applied to detect the concentrations of interleukin (IL)-17, IL-6, IL-10, and transforming growth factor (TGF)-β_1 in the peripheral blood. The therapy of hCG combined with IG may induce a decrease in the Th17/Treg ratio and the Treg bias, which may be beneficial for the maintenance of pregnancy. The levels of Th17 cells and related cytokines IL-17 and IL-6 decreased after the treatment ($P<0.05$). At the same time, levels of Treg cells and the related cytokines IL-10 and TGF-β_1 increased ($P<0.05$). The Th17/Treg ratio decreased significantly after treatment. In conclusion, the occurrence of URSA may be associated with the Th17/Treg balance disorder, and correcting the disorder may be one of the mechanisms behind the efficacy of combination therapy for URSA.

Introduction

The occurrence of two or more consecutive spontaneous miscarriages before the 20th week of gestation is defined as recurrent spontaneous abortion (RSA), a condition that affects 1-5% women of reproductive age (1). The etiology mainly involves genetic disorders, immune diseases, endocrine dysfunctions, infection and anatomic abnormalities (2). The cause of unexplained recurrent spontaneous abortion (URSA) remains unknown. However, it has been suggested that URSA is associated with the failure of fetal-maternal immunologic tolerance (3).

URSA affects approximately 50% of RSA patients, and it may be associated with the failure of fetal-maternal immunologic tolerance. Tolerance of the fetus by the maternal immune system is considered to depend on the interactions of an array of cytokines. Cytokines produced by CD4$^+$ T cells may play an important role in maternal-fetal immunoregulation (2). The CD4$^+$ T cells can be classified into the following subsets: T helper (Th)1, Th2, Th17, and regulatory T (Treg) cells according to their functions. A study indicated that altered immunity in URSA is dominated by the Th1/Th2 hypothesis (4). However, the Th1/Th2 paradigm is not enough to explain the mechanism regarding how the fetus is increased by maternal immune cells. The Th1/Th2 paradigm has been expanded to the Th1/Th2/Th17, and Treg cell paradigm. The Th17 and Treg cells were recently discovered as lymphocyte subsets with a unique differentiation and growth regulatory mechanism, which is different from Th1 and Th2 cells. These are known to play a major role in the development of autoimmune diseases and infection. Previous studies have shown that the imbalance of Th17/Treg may be associated with URSA (5-7). Large doses of immunoglobulin have been administered as immunotherapy to idiopathic RSA women (8,9). We improved the current methods by using small doses of immunoglobulin (IG) combined human chorionic gonadotropin (hCG) hormone for the therapy of URSA women with a success rate of 95.1% (10).

In this study, we aimed to investigate the effects of the combined therapy of immunoglobulin plus hCG on Th17/Treg balance in URSA women.

Materials and methods

Sample selection. The study enrolled 20 URSA patients who had at least two consecutive spontaneous miscarriages that occurred before 20 weeks gestation and had no chromosomal,

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Table I. Primer sequences, size of PCR product and annealing temperature.

Genes	Nucleotide sequences (5'-3')	Annealing temperature (˚C)	Size of PCR product (bp)
IL-17	TGTCCACCATGTGGCCTAAGAG GTCCGAAATGAGGCTGTCTTTGA	60	119
IL-6	CAAAGATGGCTGAAAAAGATGGATG GATGAACTAATTAAACCTGTGGGAG	58	313
IL-10	CTTGTCTGAGATGATCCAGTTTTAC AAGAGAAATGAGCAAGAGATCTGAC	58	298
TGF-β_1	GGGACTATCCACCTGCAAGA CCTCCTTGGCGTAGTAGTCG	55	239
β-actin	CGGGAAATCGTGCGTGACAT CGGACTCGTCATACTCCTGCTTG	62	481

IL, interleukin; TGF-β_1, transforming growth factor-β_1.

anatomic, endocrine dysfunctions, or infections of the reproductive tract. The husband's semen analysis was normal. All the subjects were patients of the Department of Obstetrics and Gynecology at the Affiliated Hospital of Xuzhou Medical College between June 2013 and April 2014. Their mean age was 26.05±2.31 years. Informed consent to participate in the study was obtained from the patients. The study was approved by the Ethics Committee of the Xuzhou Central Hospital (Jiangsu, China).

Immunoglobulin therapy and sampling time. IG-combined hCG was administered to the study subjects as soon as pregnancy was confirmed by a positive urine β-hCG test. The therapy protocol was as follows: 0.3 g IG were injected intramuscularly every three weeks until 14 weeks of gestation, and 2,000 U hCG were injected intramuscularly every two days. Ultrasound was used to detect whether the embryo was in normal development between 12 and 13 weeks of pregnancy. The dose of hCG was decreased after the 13th week of pregnancy and finally discontinued. After pregnancy, 8 ml peripheral blood was drawn before treatment and at the 12 weeks of gestation (after treatment) respectively. Samples included 5 ml of K2EDTA anticoagulant and 3 ml procoagulant. All the pregnancies were ongoing beyond 28 weeks of gestation.

Drugs and reagents. IG (Franc Group), hCG (Taibang Biological Products Co. Ltd., Shandong, China), High-purity Total RNA extraction kit (Generay Biotech Co., Ltd., Shanghai, China), TIANScript RT kit (Tiangen Biotech Co., Ltd., Shanghai, China), polymerase chain reaction (PCR) kit of 2X Taq Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), DNA molecular size marker of 100 bp DNA ladder (Tiangen Biotech Co., Ltd.) and enzyme-linked immunosorbent assay (ELISA) kit (Suzhou Calvin Biotechnology Co., Ltd., Suzhou, China) were used. Primers were produced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Total RNA preparation and cDNA synthesis. Total RNA was isolated from EDTA anticoagulated blood using High-purity Total RNA Extraction kit (Generay Biotech Co., Ltd.). Synthesis of cDNA was performed using TIANScript RT kit (Tiangen Biotech Co., Ltd.) with a 20-μl reaction system. The whole process was carried out on ice.

PCR. Amplification reactions (25 μl) consisted of 2 μl of cDNA, 12.5 μl of 2X Taq Master Mix, 8.5 μl of ddH_2O, 1 μl of forward primer and 1 μl of reverse primer. PCR reaction was performed as follows: An initial denaturation step of 94˚C for 5 min, followed by 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 60 sec for 35 cycles and a final extension at 72˚C for 10 min. Primer sequences, the size of PCR product and annealing temperature are described in Table I. PCR products were observed on a 2% agarose gel that was stained with ethidium bromide, electrophoresed for 1.5 h at 40 mA and photographed under ultra-violet light. The OD ratio of target genes and β-actin was calculated to measure the mRNA levels of target genes relatively.

ELISA to detect cell factor. The absorbance of serum interleukin (IL)-17, IL-6, IL-10 and transforming growth factor (TGF)-β_1 were detected using the ELISA kit, the concentration was calculated in accordance with the absorbance value.

Separation of PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (MP Biomedicals, Solon, OH, USA) density centrifugation. After washing with Hanks' balanced salt solution, the cells were adjusted to a final concentration of $1x10^7$ cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (all from Gibco, Grand Island, NY, USA). Prepared PBMCs were stored at 4˚C in the dark.

Flow cytometry. To activate PBMCs, 1 ml of $1x10^7$/ml of cell suspension was incubated with 10 ng/ml PMA and 0.5 mM ionomycin (eBioscience, San Diego, CA, USA) for 5 h at 37˚C in a 5% CO_2 humidified incubator. Monensin (1 μl of a x1,000 solution) (eBioscience) was also applied to enhance intracellular cytokine staining. After incubation, PBMCs were washed

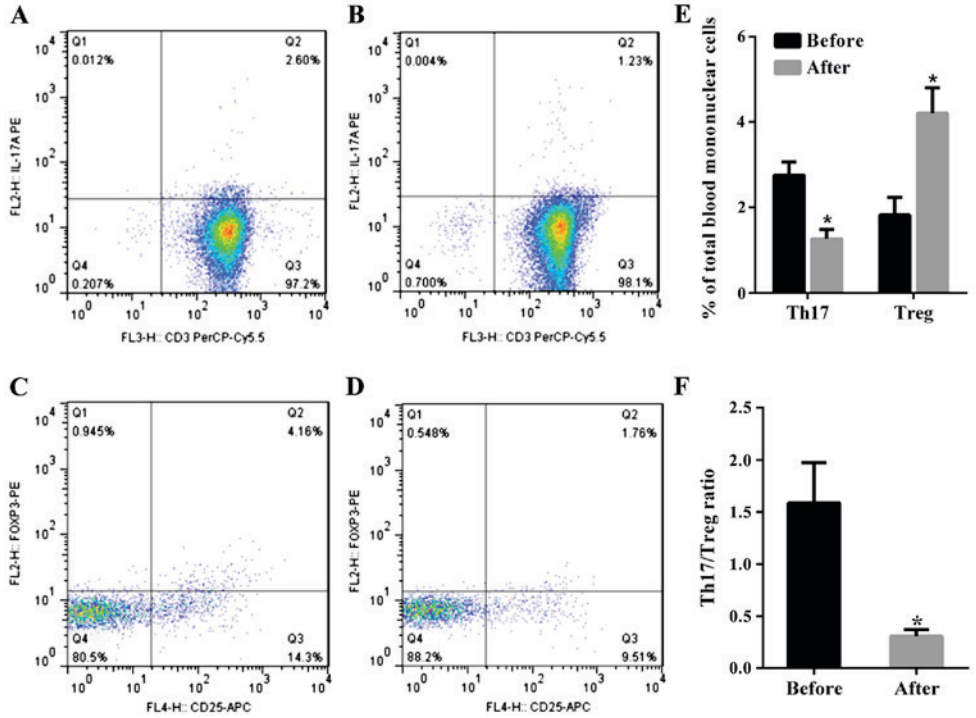

Figure 1. The percentage of T helper 17 (Th17) and regulatory T (Treg) cells in peripheral blood mononuclear cells (PBMCs) and the Th17/Treg ratio varies in patients with a successful pregnancy after immunotherapy. The percentage of Th17 and Treg cells in PBMCs were detected by flow cytometry in patients with unexplained recurrent spontaneous abortion (URSA) before and after therapy. Representative $CD3^+CD8^+IL\text{-}17A^+$ flow cytometry plots from patients with URSA are shown (A) before and (B) after therapy. Representative $CD4^+CD25^+Foxp3^+$ flow cytometry plots from patients with URSA are shown (C) before and (D) after therapy. (E) The percentage of Th17 cells in PBMCs significantly decreased after therapy. The percentage of Treg cells in PBMCs significantly increased after therapy ($^*P<0.01$, paired t-test). Bar represents the Th17 and Treg cell frequency and the means ± SEM are indicated. (F) The Th17/Treg ratio in PBMCs significantly decreased after therapy ($P<0.01$, paired t-test). Bar represents the frequency and the means ± SEM are indicated.

in phosphate-buffered saline (PBS) with 0.09% (w/v) sodium azide (eBioscience) twice. This was followed by staining with the anti-CD3-FITC and anti-CD8-APC (both from eBioscience), and then incubation for 15 min at 4˚C. The cells were washed in PBS and fixed with fixation buffer (eBioscience). The cells were washed twice with 1X permeabilization buffer and incubated with 0.25 μg conjugated anti-human IL-17A-PE (both from eBioscience) at room temperature for 20 min. After intracellular staining, the cells were washed with 1X permeabilization buffer and resuspended in 0.5 ml of permeabilization buffer. The proportion of IL-17-producing T cells in peripheral blood lymphocytes were enumerated by flow cytometry.

To identify Treg cells, PBMCs were stained with 0.25 μg FITC-conjugated rabbit monoclonal CD4 antibody (dilution, 1:50; cat. no. 85-11-0048-42) and 0.25 μg APC-conjugated rabbit monoclonal CD25 antibody (dilution, 1:50; cat. no. 85-17-0259-42) for surface antigens, and 0.25 μg PE-conjugated rabbit monoclonal Foxp3 antibody (dilution, 1:50; cat. no. 85-12-4776-42), all purchased from eBioscience (San Diego, CA, USA), for intracellular molecules, as per the manufacturer's instructions. PBMC ($1x10^6$) was washed twice in PBS following staining with the fluorochrome-conjugated antibodies specific for cell surface antigen markers for 20 min in the dark at 4˚C. In order to stain the intracellular molecule, Foxp3, cells were permeabilized with permeabilization/fixation buffer and stained with anti-Foxp3 antibody following the surface staining. PE-Rat IgG2a was used as an isotype control for anti-Foxp3-PE antibody. Cells were resuspended in 0.5 ml of staining buffer for subsequent flow cytometry analysis. The prepared cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). CellQuest Pro software (BD Biosciences) was used for data analysis.

Statistical analysis. Statistical analysis was performed using the SPSS 16.0 statistical program. Numerical data are presented as mean ± SEM. To compare the results of immunologic studies before and after hCG plus immunoglobulin treatment, paired t-test was applied. Correlations between IL-17 and IL-6, IL-10 and TGF-β_1 were performed using Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Th17 cells, Treg cells and the ratio of Th17/Treg cells in patients with URSA after the treatment of IG plus hCG. The percentage of Th17 cells ($CD3^+CD8^+IL\text{-}17A^+$T cells) in PBMC in the total patient population with URSA before therapy was 2.76±0.31%, and the percentage of this subset after therapy was 1.527±0.22% ($P<0.01$, paired t-test). Thus, the percentage of Th17 cells significantly decreased after therapy compared to before therapy (Fig. 1). The percentage of Tregs ($CD4^+CD25^+Foxp3^+$ T cells) in PBMC significantly increased in patients with URSA following therapy at 4.21±0.59% as compared to before at 1.82±0.42% therapy ($P<0.01$, paired t-test). Thus, the percentage of Tregs significantly increased after therapy (Fig. 1). The mean Th17/Treg ratio in all

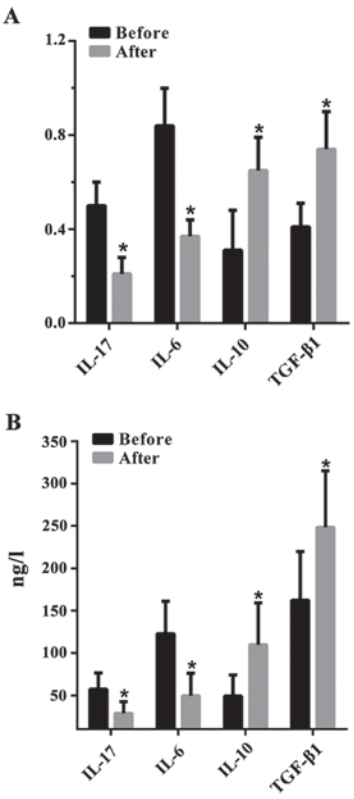

Figure 2. (A) RT-PCR and (B) enzyme-linked immunosorbent assay (ELISA) results of interleukin (IL)-17, IL-6, IL-10 and transforming growth factor-β_1 (TGF-β_1) in the peripheral blood of unexplained recurrent spontaneous abortion (URSA) patients before and after therapy. The concentrations of Th17-type cytokines IL-17 and IL-6 were significantly decreased in the serum of patients with URSA after therapy (*P<0.01, paired t-test). Treg-type cytokines such as IL-10 and TGF-β1 were significantly increased after therapy (*P<0.01, paired t-test). Values are expressed as the mean ± SEM.

patients with URSA before immunotherapy was 1.57 but was reduced to 0.36 after immunotherapy (P<0.01). Therefore, the mean Th17/Treg ratio significantly decreased after therapy compared to the ratio before therapy (P<0.01, paired t-test), as shown in Fig. 1.

The levels of IL-17, IL-6, IL-10 and TGF-β_1 in the peripheral blood of URSA patients before and after therapy. The concentration of Th17-type cytokines IL-17 and IL-6 was significantly decreased in the serum of patients with URSA after therapy [0.21±0.07 and 0.37±0.07 (RT-PCR); 28.84±13.61 and 49.39±26.72 ng/l (ELISA)] (P<0.01, paired t-test) than before therapy [0.50±0.10 and 0.84±0.16 (RT-PCR); 57.17±19.31 and 122.66±38.32 ng/l (ELISA)]. Levels of Treg-type cytokines such as IL-10 and TGF-β_1 significantly increased after therapy [0.65±0.14 and 0.74±0.16 (RT-PCR); 109.64±49.65 and 248.17±66.91 ng/l (ELISA)] than before therapy [0.31±0.17 and 0.41±0.10 (RT-PCR); 48.93±25.15 and 162.05±57.67 ng/l (ELISA)] (P<0.01, paired t-test) (Fig. 2).

Discussion

Th17 cells and Treg cells are recently discovered lymphocyte subsets, that are different from Th1 and Th2. Th17 cells are associated with chronic inflammation through the secretion of the inflammatory cytokines IL-17A (also known as IL-17), IL-17F, IL-6, IL-21, IL-22 and TNF-α, which play a major role in the development of organ transplant rejection (11,12). Normal pregnancy is similar to an allograft. Nakashima *et al* found that there were lower levels of Th17 cells in the peripheral blood of normal pregnant women (13). Recent studies reported that the proportions and concentrations of Th17 cells in the peripheral blood were increased in pregnant women with URSA than when compared to normal pregnant and non-pregnant women (6). This association correlated with the secretion of other inflammatory cytokines (14). To study the expression of IL-17 in women that have undergone abortion, we suggested that the activation of IL-17 could increase the expression of NF-κB, thereby reducing the number of progesterone receptors and weakening its function. Therefore, progesterone cannot combine with a sufficient number of progesterone receptors, resulting in decidua dysplasia and inadequate nutrition for the embryo, finally leading to miscarriage (15). Treg cells comprise a subset of T-lymphocytes with pivotal immunological regulation effects in maintaining a stable internal environment and inducing immune tolerance to graft by inhibiting effector T-cell responses and conventional T-cell activation by promoting the secretion of suppressive cytokines (16). The role of Treg cells in immune tolerance is still unclear. Currently, Treg cells indicate immunosuppression by secreting immunosuppressive factors, such as IL-10 and TGF-β_1, that contribute to inhibition of antigen presenting cells and antagonism of effector T cells (17). Several studies have shown that proportions of Treg cells in the peripheral blood and decidua of pregnant women with URSA were decreased when compared to normal pregnant women; they also presented with lower immunosuppressive activity than normal pregnant women (18,19). The adoptive transfer of pregnancy-induced $CD4^+CD25^+$ Treg therapy contributed to successful pregnancy and reduced the rate of spontaneous abortion of abortion-prone mice (20).

Our previous studies demonstrated that hCG inhibited the expression of TNF-α and INF-γ of maternal-fetal interface and reduced the embryonic resorption rate of abortion-prone mice (21). Bai *et al* cultured human PBMCs with different doses of hCG and found that hCG had significantly suppressed the effect on IL-6 and TNF-α mRNA expression, indicating that hCG could inhibit the production of pro-inflammatory cytokines (22). In the presence of IL-6, $CD4^+$ T cells differentiated to Th17 cells by TGF-β_1, resulting in autoimmunity and inflammation (23). Therefore, hCG can inhibit the differentiation of Th17 cells and increase the differentiation of Treg cells by inhibiting the expression of IL-6. Schumacher *et al* suggested that levels of hCG and Treg in the decidua and placenta of pregnant women with RSA were lower than those in normal pregnant women (24). Levels of hCG and Treg were positively correlated and hCG could upregulate the LH/CG receptor on the surface of Treg and attract Treg cells into the fetal-maternal interface, inducing the formation of immune tolerance state (24).

Immunoglobulin (IG) has been administered as immunotherapy to URSA women (8,25-29). Although the therapeutic effect of IG is controversial, recent meta-analysis of IG treatment revealed that IG has significantly higher success rate in women with immune abnormalities when compared to women without any immune abnormalities (30). The therapeutic effect

of IG was achieved mainly by the injection of IgG into the body. Maddur *et al* found that Th17 cells and their effector cytokines were significantly decreased after the addition of IG into cultured T cells, suggesting that IG could inhibit the differentiation and amplification of Th17 cells, as well as the production of their effector cytokines. IG was also able to significantly enhance Treg cells among memory T cells (31). In this study, we investigated the levels of Th17 and Treg as well as the related cytokines in the peripheral blood of pregnant women with URSA before and after treatment. We found that, after the treatment of IG plus hCG, IL-17^{+} T cells and the related cytokines IL-17 and IL-6 levels were lower than before. However, Foxp3^{+} T cells and the related cytokines IL-10 and TGF-β_1 levels were increased. This suggests that the therapy of immunoglobulin combined hCG is able to inhibit the differentiation of Th17 cells and increase the differentiation of Treg, thereby making the Th17/Treg paradigm into the Treg immune bias, inducing maternal immune tolerance to the embryo and thereby promoting the development of full-term pregnancy. This study also indicates that correction of the disorder of Th17/Treg balance may be one of the mechanisms through which immunoglobulin combined hCG can be used to treat URSA. Our previous clinical and animal studies have shown that the therapeutic effect of immunoglobulin plus hCG was superior to monotherapy (10,21). However, the mechanisms of combination therapy are still under exploration.

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