

Th17/Treg-cell balance in the peripheral blood of pregnant females with a history of recurrent spontaneous abortion receiving progesterone or cyclosporine A

SONGCUN WANG^{1,2*}, MENGDI LI^{1,2*}, FENGRUN SUN^{1,2}, CHUNQIN CHEN^{1,2},
JIANGFENG YE², DAJIN LI^{1,2}, JINFENG QIAN^{1,2} and MEIRONG DU^{1,2}

¹NHC Key Laboratory of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research);

²Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200080, P.R. China

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Abstract. A successful pregnancy requires the maternal immune system to accept a fetus expressing allogeneic paternal antigens and provide competent responses to infections. Accordingly, maternal-fetal immune abnormalities may have an important role in the development of recurrent spontaneous abortion (RSA). Ever since the establishment of the association between immunologic abnormalities and RSA, various types of immune therapy to restore normal immune homeostasis have been increasingly developed. Although previous studies have focused on the maternal-fetal interface, non-invasive examination is of great importance in clinical practice. The present study investigated the balance between type-17 T-helper (Th17) and T-regulatory (Treg) cells in the peripheral blood to improve the current understanding of the pathogenesis of RSA. Imbalances in Th17/Treg cells and associated molecular profiles were observed in patients with RSA. Furthermore, it was determined that the immunosuppressant cyclosporine A reduced the proportion of Th17 cells and promoted Treg-cell dominance by upregulating the expression of co-inhibitory molecules in pregnant females with a history of RSA. Progesterone, the traditional maternal-care drug, also had a certain immunomodulatory role through restoring the levels of several co-inhibitory molecules (including T-cell immunoglobulin mucin family member-3, programmed cell

death-1 and cytotoxic T-lymphocyte associated protein-4) in the treatment of RSA. Changes in these immune molecules within the maternal peripheral blood may be indicators for monitoring pregnancy and prediction of RSA.

Introduction

Recurrent spontaneous abortion (RSA), defined as two or more consecutive pregnancy losses prior to 20 weeks of gestation, may seriously compromise not only the physical but also psychological well-being of women. Although parental or embryonic karyotype anomalies, infection-associated factors, endocrine disorders, uterine anatomic abnormalities and anti-phospholipid syndrome have been acknowledged as the common causes of RSA, approximately half of the cases still remain unexplained (1). The development of the allogeneic fetus in the maternal uterus represents an immunological paradox. A successful pregnancy requires the maternal immune system to accept the fetus expressing allogeneic paternal antigens and provide competent responses to infections. Thus, maternal-fetal immune abnormalities might have an important role in the development of RSA (2).

The modulation of the maternal CD4⁺ T-cell response to fetal antigens has been considered an important component of maternal-fetal tolerance during pregnancy. Upon encountering antigens on the surface of antigen-presenting cells or being driven by a set of cytokines, naive CD4⁺ T cells are able to differentiate into distinct subsets, including type-1 T-helper (Th1), Th2, Th17 and T-regulatory (Treg) cells (3). While a pro-inflammatory microenvironment is required for trophoblastic growth and invasion (4), Th1 cytokines, including tumor necrosis factor- α and interferon- γ , have deleterious effects on pregnancy (5). For numerous years, it has been thought that a shift in the maternal immune response toward a Th2 bias is crucial for maintaining a successful pregnancy (6).

Th17 cells have a critical role in inducing inflammation, while abnormal Th17-cell levels have been associated with the pathogenesis of autoimmune diseases (7). Treg cells express anti-inflammatory cytokines, including interleukin (IL)-10 and transforming growth factor (TGF)- β 1, which dampen an

Correspondence to: Dr Meirong Du or Dr Jinfeng Qian, NHC Key Laboratory of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research), Fudan University Shanghai Medical College, 1326 Pingliang Road, Shanghai 200080, P.R. China
E-mail: dmrlq1973@sina.cn
E-mail: qjf2003@163.com

*Contributed equally

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excessively effective immune response (8). Recent studies have indicated that the balance between Treg and Th17 cells is also important for maintaining a normal pregnancy, while a shift in the Th17/Treg ratio toward Th17 cells has been suggested to cause several pregnancy-associated diseases, including RSA, pre-eclampsia and gestational diabetes mellitus (9-11).

In addition to the signal from antigen recognition, T-cell activation requires a second signal from co-stimulatory molecules, which maybe either positive or negative (12,13). Among the inhibitory co-stimulatory molecules, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin mucin-3 (Tim-3) and programmed cell death-1 (PD-1) have attracted much attention regarding their roles in regulating Th17- and Treg-cell function to maintain a balanced immune response (14-17). For instance, Tim-3 has been associated with a shift in the balance from Th17 and Treg cells to Treg dominance (16). The PD-1 signaling pathway has been demonstrated to have an important role in Th17-cell function during pregnancy (18). Furthermore, PD-1 and CTLA-4 are critical for the suppressive activity of Treg cells (19,20), while PD-1⁺Tim-3⁺Treg cells exhibit an increased effector function (21). Previous studies by our group revealed that the expression of these three molecules was lower in human miscarriages than in normal pregnancies, while their blockade during the implantation window increased maternal rejection of the fetus in CBA/Jx BALB/c matings (22,23).

Progesterone (P4), the most common medication for fetal protection in RSA, is a multifunctional female hormone that promotes breast development, ovulation and pregnancy maintenance; it is mainly secreted by ovarian granulosa cells and the corpus luteum during non-pregnancy and by the placenta during pregnancy (24). While the mechanisms remain to be fully elucidated, P4 has been reported to dampen immune responses to fetal and maternal antigens (25). As an immunosuppressant, cyclosporine A (CsA) has been widely used in organ transplantation (26). CsA not only induces Th2 immuno-dominance at the maternal-fetal interface, but also promotes trophoblastic proliferation and invasion, further promoting placental formation (27). This immunosuppressive agent, categorized as pregnancy category C by the Food and Drug Administration, has been utilized for the treatment of RSA at our hospital (Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College) since 2005.

To the best of our knowledge, studies on the effects of P4 or CsA on the proportion of Th17 and Treg cells within the peripheral blood of patients with RSA are rare. Therefore, the present study aimed to investigate the effects of P4 or CsA therapy on the Th17/Treg cell balance and the expression of associated molecules in the peripheral blood of pregnant females with a history of RSA.

Materials and methods

Study population. For the present study, subjects aged between 20 and 39 years were recruited at the Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China) between July 2018 and December 2018. The clinical characteristics of the enrolled subjects are summarized in Table I. The cohort included 30 females with normal early pregnancy (NP group),

25 with RSA (RSA group), 27 pregnant females with a history of RSA receiving progesterone (P4 group) and 24 pregnant females with a history of RSA receiving cyclosporine A (CsA group). Subjects with RSA included those undergoing spontaneous abortion and who also had a history of two or more consecutive spontaneous abortions before 20 weeks (the mean pregnancy week was 6.28 ± 0.09) of gestation without known causes (including parental or embryonic karyotype anomalies, uterine anatomic abnormalities, infection-associated factors, endocrine disorders and antiphospholipid syndrome). The subjects in the NP group (the mean pregnancy week was 6.26 ± 0.11) had at least one successful pregnancy and no history of spontaneous abortions. None of the subjects had any history of autoimmune diseases or immunotherapy, renal or liver diseases, alcohol addiction, smoking or vaccination within 3 months prior to consultation. Approval of the present study was obtained from the ethics committee of the Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China) and all subjects provided written informed consent.

In this open-label (i.e. non-blind) study, pregnant females with a history of RSA were randomly allocated to the P4 or CsA group after a positive human chorionic gonadotropin urine test. The P4 group received dydrogesterone (Abbott) 10 mg Q8 h orally, while the CsA group received CsA (Sanofi) 50 mg Q8 h orally. After 10 days, venous blood was collected (at this time the mean pregnancy week of P4 group was 6.16 ± 0.1 , the mean pregnancy week of CsA group was 6.0 ± 0.09).

Separation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated from 5 ml heparinized venous blood using Ficoll-Hypaque density gradient centrifugation ($573 \times g$ at room temperature for 20 min). Cells were collected at the interface and washed with PBS. Cells were cultured in RPMI 1640 (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 1 $\mu\text{g/ml}$ amphotericin B. Phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; BioLegend, Inc.), ionomycin (1 $\mu\text{g/ml}$; BioLegend, Inc.) and brefeldin A (10 mg/ml; BioLegend, Inc.) were added to the culture for 4 h prior to intracellular cytokine analysis.

Flow cytometric analysis. Cell surface molecular expression and intracellular cytokine production were evaluated using flow cytometry (FCM). Alexa eFluor[®] 488-conjugated anti-human forkhead box p3 (Foxp3; cat. no. 320112), phycoerythrin (PE)-conjugated anti-human CD366 Tim-3 (cat. no. 345006) or CD152 CTLA-4 (cat. no. 349906), PE/cyanine 7-conjugated anti-human IL-17A (cat. no. 512315), allophycocyanine-conjugated anti-human CD279 PD-1 (cat. no. 329908) or LAP TGF- β 1 (cat. no. 349608), Brilliant Violet 421-conjugated anti-human IL-10 (cat. no. 501422) and Brilliant Violet 510-conjugated anti-human CD4 (cat. no. 317444) antibodies (BioLegend, Inc.) were used. For intracellular staining, cells were fixed and permeabilized using the Fix/Perm kit (cat. no. 421401/2). The fix/perm kit and all antibodies aforementioned were purchased from BioLegend, Inc. Flow cytometry was performed on a Beckman-Coulter CyAn ADP cytometer and the results were analyzed using FlowJo software (version 7.6; FlowJo, LLC).

Table I. Clinical characteristics of enrolled subjects.

Subjects ^b	NP (n=30)	RSA (n=25)	P4 (n=27)	CsA (n=24)	P-value
Age mean (years) ^a	28.07±0.86	28.96±0.98	29.26±0.83	29±1.0	0.80
Age range (years)	20-39	22-38	23-38	21-39	N/A
Previous spontaneous abortions ^a	-	2.92±0.22	2.85±0.15	2.95±0.23	0.93
Pregnancy week (HCG urine test) ^a	-	-	4.72±0.09	4.58±0.09	0.33
Pregnancy week (venous blood was collected) ^a	6.26±0.11	6.28±0.09	6.16±0.1	6.0±0.09	0.31

^aValues are expressed as the median ± standard error of the mean. RSA was defined as spontaneous abortion in patients who also had a history of two or more consecutive spontaneous abortions before 20 weeks of gestation without known causes. ^bNone of the subjects had any history of treatment. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. N/A, not applicable; CsA, cyclosporin A; RSA, recurrent spontaneous abortion; P4, progesterone; HCG, human chorionic gonadotropin.

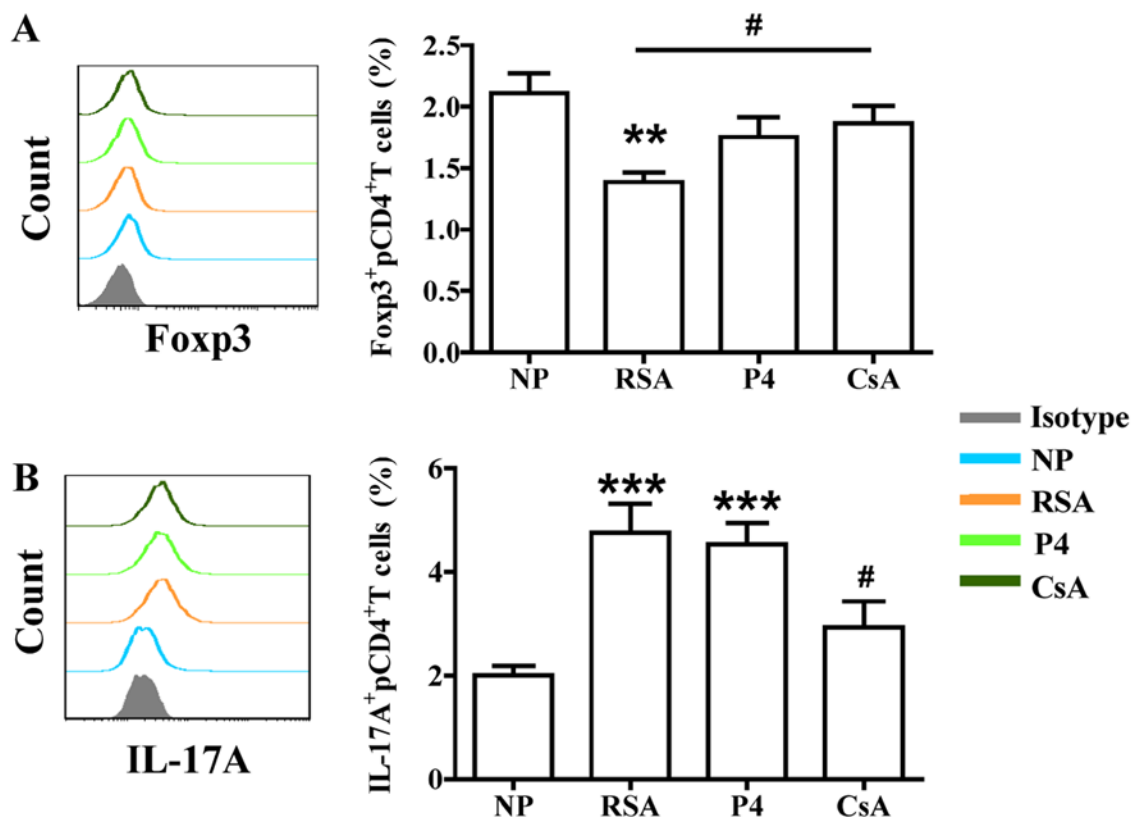


Figure 1. Effects of P4 and CsA on the percentage of Treg and Th17 cells in peripheral blood. (A) Compared with that in the NP group, the subjects in the RSA group had a significantly lower percentage of Treg cells in the peripheral blood. CsA significantly increased the percentage of Treg cells. (B) Compared with that in the NP group, the subjects in the RSA group had a higher percentage of Th17 cells in the peripheral blood. CsA decreased the percentage of Th17 cells. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. **P<0.01, ***P<0.001 vs. NP; #P<0.05 vs. RSA. CsA, cyclosporine A; Treg cells, T-regulatory cells; Th17 cells, type-17 T-helper cells; RSA, recurrent spontaneous abortion; Fox, forkhead box; IL, interleukin; P4, progesterone.

Statistical analysis. All variables were normally distributed in this study. Thus, variables were presented as means and standard deviation (SD). One-way ANOVA was used to evaluate differences. P<0.05 was considered to indicate a statistically significant difference. In cases where P<0.05 after ANOVA, Bonferroni's post hoc test was performed to determine differences between each group. All analyses were carried out using the GraphPad Prism 7 software (GraphPad Software, Inc.).

Results

CsA significantly increases the percentage of Treg cells and decreases the percentage of Th17 cells in the peripheral blood of pregnant females with a history of RSA. As presented in Fig. 1, the patients from the RSA group had a significantly decreased percentage of Treg cells (CD4⁺Foxp3⁺ cells) and an increased percentage of Th17 cells (CD4⁺IL-17A⁺ cells) in the peripheral blood compared with those in the NP group.

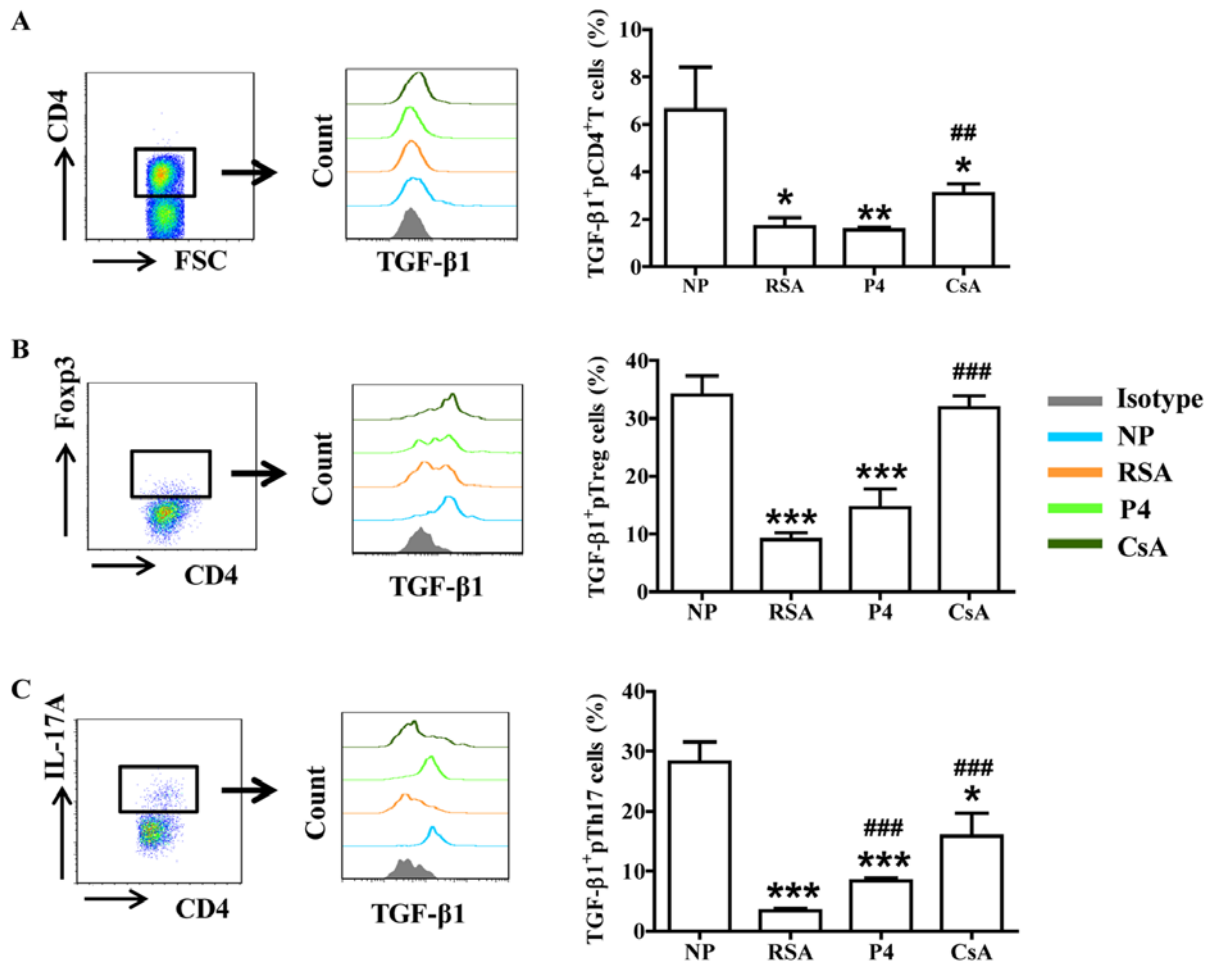


Figure 2. CsA promotes TGF- β 1 expression in peripheral blood cells of pregnant females with RSA. Compared with that in the NP group, the production of TGF- β 1 in (A) CD4⁺ T cells, (B) Treg cells and (C) Th17 cells in the peripheral blood of subjects in the RSA group was decreased. While CsA and P4 promoted the expression of TGF- β 1 in Th17 cells, P4 had no effect on TGF- β 1 production by CD4⁺ T cells and Treg cells. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NP; ## $P < 0.01$, ### $P < 0.001$ vs. RSA. CsA, cyclosporine A; pTreg cells, peripheral T-regulatory cells; Th17 cells, type-17 T-helper cells; RSA, recurrent spontaneous abortion; P4, progesterone; Fox, forkhead box; IL, interleukin; TGF, transforming growth factor; FSC, forward scatter.

Of note, CsA increased the percentage of Treg cells, while decreasing the percentage of Th17 cells in the peripheral blood of pregnant females with a history of RSA compared with the percentages in the RSA group. However, P4 exhibited no significant effect on the proportion of Th17 or Treg cells in pregnant females with a history of RSA compared with the proportions in the RSA group.

CsA promotes TGF- β 1 and IL-10 production in peripheral blood cells of pregnant females with a history of RSA. To assess whether CsA or P4 regulates the biological functions of Th17/Treg cells in pregnant females, the production of cytokines (associated with Treg- and Th17-cell function) by peripheral T cells was first analyzed. Compared with those in the NP group, patients in the RSA group exhibited significantly lower TGF- β 1 and IL-10 expression in total peripheral (p) CD4⁺ T cells, pTreg cells and pTh17 cells. However, CsA increased TGF- β 1 and IL-10 expression in the peripheral blood cells of pregnant females with a history of RSA, whereas P4 promoted only TGF- β 1 expression in pTh17 cells (Figs. 2 and 3).

Effects of CsA and P4 on CTLA-4, Tim-3 and PD-1 expression in peripheral blood cells of pregnant females with a history of RSA. In addition to the signal from the T-cell receptor complex after antigen recognition, T-cell activation requires a second signal from co-signaling molecules. Recently, the role of co-signaling molecules, particularly co-inhibitory molecules, in maternal-fetal immunity has been explored (28). The effects of CsA and P4 on CTLA-4, Tim-3 and PD-1 expression in the peripheral blood cells of pregnant females were then assessed. As presented in Figs. 4-6, the patients in the RSA group demonstrated significantly lower CTLA-4, Tim-3 and PD-1 expression in the peripheral blood cells, but the expression of Tim-3 and PD-1 remained unchanged on Th17 cells, compared with those in the NP group. CsA increased CTLA-4 expression in total pCD4⁺ T cells, pTreg cells and pTh17 cells, whereas P4 only promoted CTLA-4 expression in total pCD4⁺ T cells and pTh17 cells of pregnant females with a history of RSA (Fig. 4). CsA and P4 increased Tim-3 expression on total pCD4⁺ T cells, pTreg cells and pTh17 cells (Fig. 5). Furthermore, P4 increased PD-1 expression in pTreg cells, while CsA had no effect on PD-1 expression in peripheral blood cells (Fig. 6).

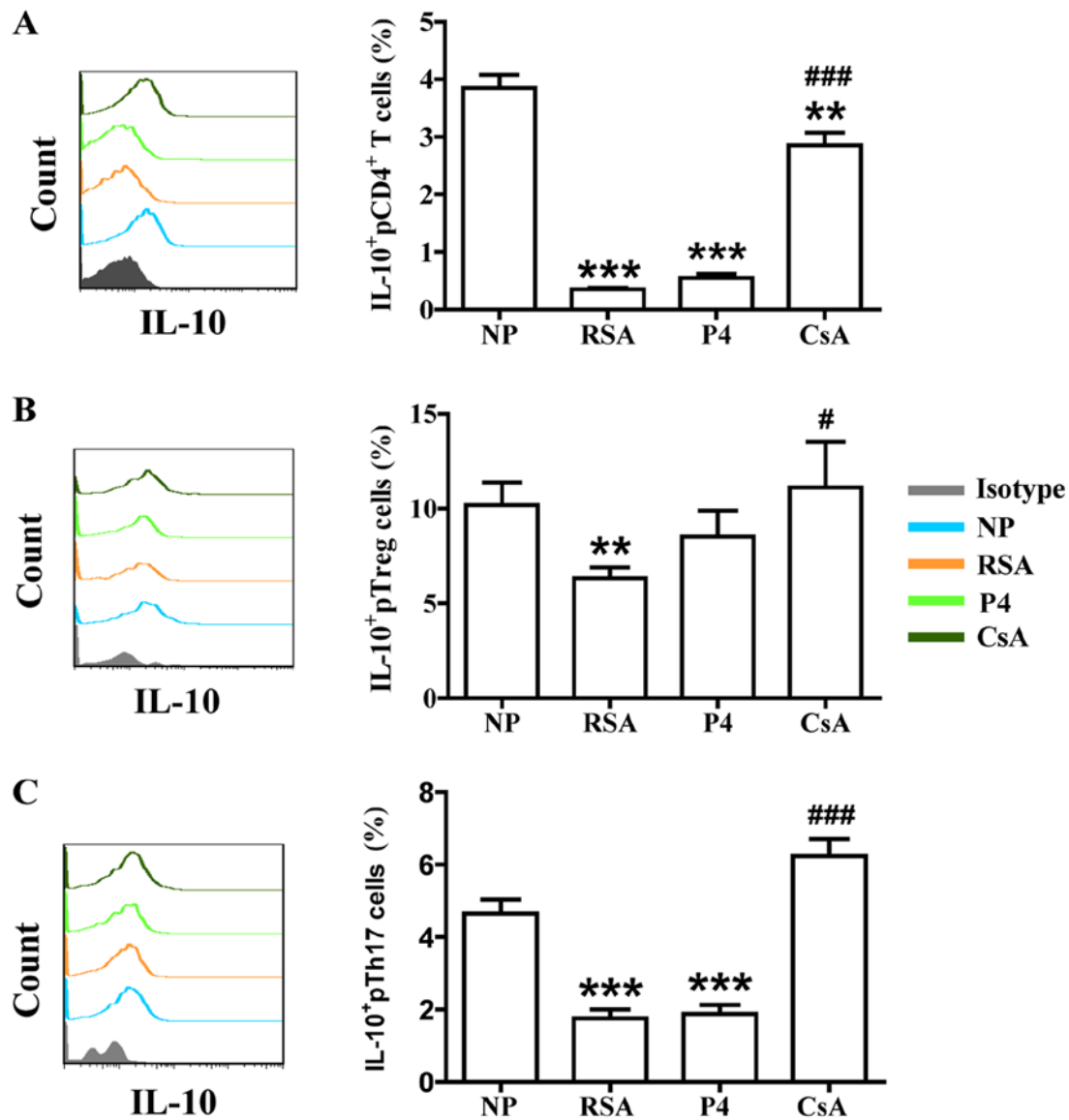


Figure 3. CsA promotes IL-10 expression in peripheral blood cells of pregnant females with RSA. Compared with that in the NP group, the production of IL-10 in (A) CD4⁺ T cells, (B) Treg cells and (C) Th17 cells in the peripheral blood of subjects in the RSA group was decreased. While CsA promoted the production of IL-10, P4 had no effect on it. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. **P<0.01, ***P<0.001 vs. NP; #P<0.05, ###P<0.001 vs. RSA. CsA, cyclosporin A; pTreg cells, peripheral T-regulatory cells; Th17 cells, type-17 T-helper cells; RSA, recurrent spontaneous abortion; P4, progesterone; IL, interleukin.

Discussion

As a semi-allograft, the human fetus is antigenically foreign to the mother. Accordingly, a successful pregnancy relies on the fine maternal-fetal immune balance between immune tolerance, allowing allogeneic fetal trophoblasts to invade maternal tissues and immune defense against a variety of pathogens. Disruption of this immune balance is thought to be associated with several pregnancy-associated complications, including RSA, preeclampsia and fetal intrauterine growth restriction (2). The present study also confirmed that imbalance of Th17/Treg cells and associated molecule is linked to RSA.

P4, the traditional treatment for RSA, inhibits uterine contraction and improves luteal-phase support and utero-placental circulation (29). Chien *et al* (30) indicated that P4 may also regulate immunity and inhibit T-cell activation

through a non-classical steroid receptor pathway during pregnancy. In early 1995, Piccinni *et al* (31) proposed that P4 was beneficial to Th2 differentiation of CD4⁺ T cells. Furthermore, Mjosberg *et al* (32) revealed that P4 regulated Treg-cell functions in human pregnancy, while Lee *et al* (33) reported that P4 inhibited Th17-cell differentiation and reduced the expression of associated functional molecules, including IL-17A. However, the present study revealed no significant differences in the proportion of Treg and Th17 cells in the peripheral blood between the P4 and RSA groups. This may be due to differences in the gestational stages tested and the fact that certain studies were performed using established cell clones.

While certain studies have suggested the involvement of Th17 cells in pregnancy-associated pathologies (34), a study by our group (35) and Wu *et al* (36) suggested that Th17 cells serve important roles in regulating trophoblast

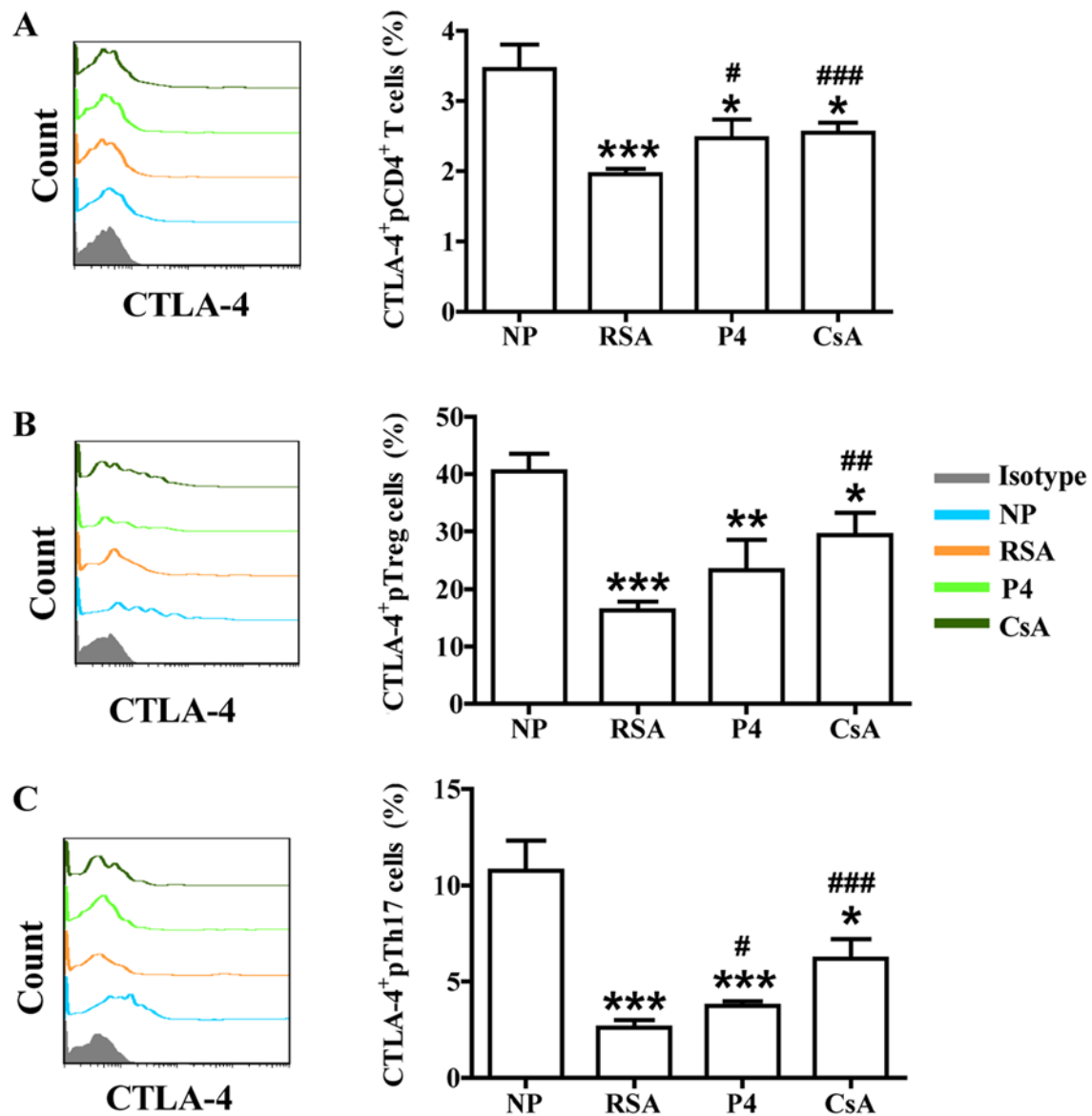


Figure 4. Effects of P4 and CsA on CTLA-4 expression in peripheral blood cells of pregnant females with a history of RSA. Compared with that in the NP group, the production of CTLA-4 in (A) CD4⁺ T cells, (B) Treg cells and (C) Th17 cells in the peripheral blood of subjects in the RSA group was decreased. CsA promoted the expression of CTLA-4 in all three indicated subsets of T cells. P4 also promoted CTLA-4 expression in total CD4⁺T and Th17 cells. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. NP; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs. RSA. CsA, cyclosporine A; pTreg cells, peripheral T-regulatory cells; Th17 cells, type-17 T-helper cells; RSA, recurrent spontaneous abortion; P4, progesterone; CTLA, cytotoxic T-lymphocyte-associated protein 4.

function. It has also been indicated that not all Th17 cells are pro-inflammatory, given that Th17 subpopulations with diverse functions may exist (37). IL-10 has been previously associated with non-pathogenic Th17 cells (38), while TGF- β 1 may be produced mainly by Th17 cells (39). Although P4 had no effect on IL-10 expression in all three indicated subsets of T cells and TGF- β 1 expression in pCD4⁺ T cells and pTreg cells, it significantly increased the expression of TGF- β 1 and CTLA-4 in pCD4⁺ T cells and pTh17 cells, as well as on Tim-3 expression in all three indicated subsets of T cells and PD-1 expression in pTreg cells. As co-stimulatory signals have important roles in T-cell function and pregnancy maintenance (18,28), despite the lack of evidence regarding improved outcomes following first-trimester progesterone therapy in females with a history of RSA (40,41), the results of

the present study suggested that P4 may also have an immunomodulatory role through certain co-inhibitory molecules (including Tim-3, PD-1 and CTLA-4) in the treatment of RSA.

The observed association between immunologic abnormalities and RSA has led to the development and use of various types of immune therapy with the objective of restoring normal immune homeostasis during pregnancy. CsA is a previously known immunosuppressive drug that has been used for the treatment of RSA based on previous studies (27,42-48). Previous studies report that CsA: i) Induced Th2 dominance and Treg-cell proliferation during the implantation window and significantly reduced the embryo absorption rate (27,42); ii) downregulated CD28 expression at the maternal-fetal interface and upregulated CTLA-4 expression in abortion-prone mice (43);

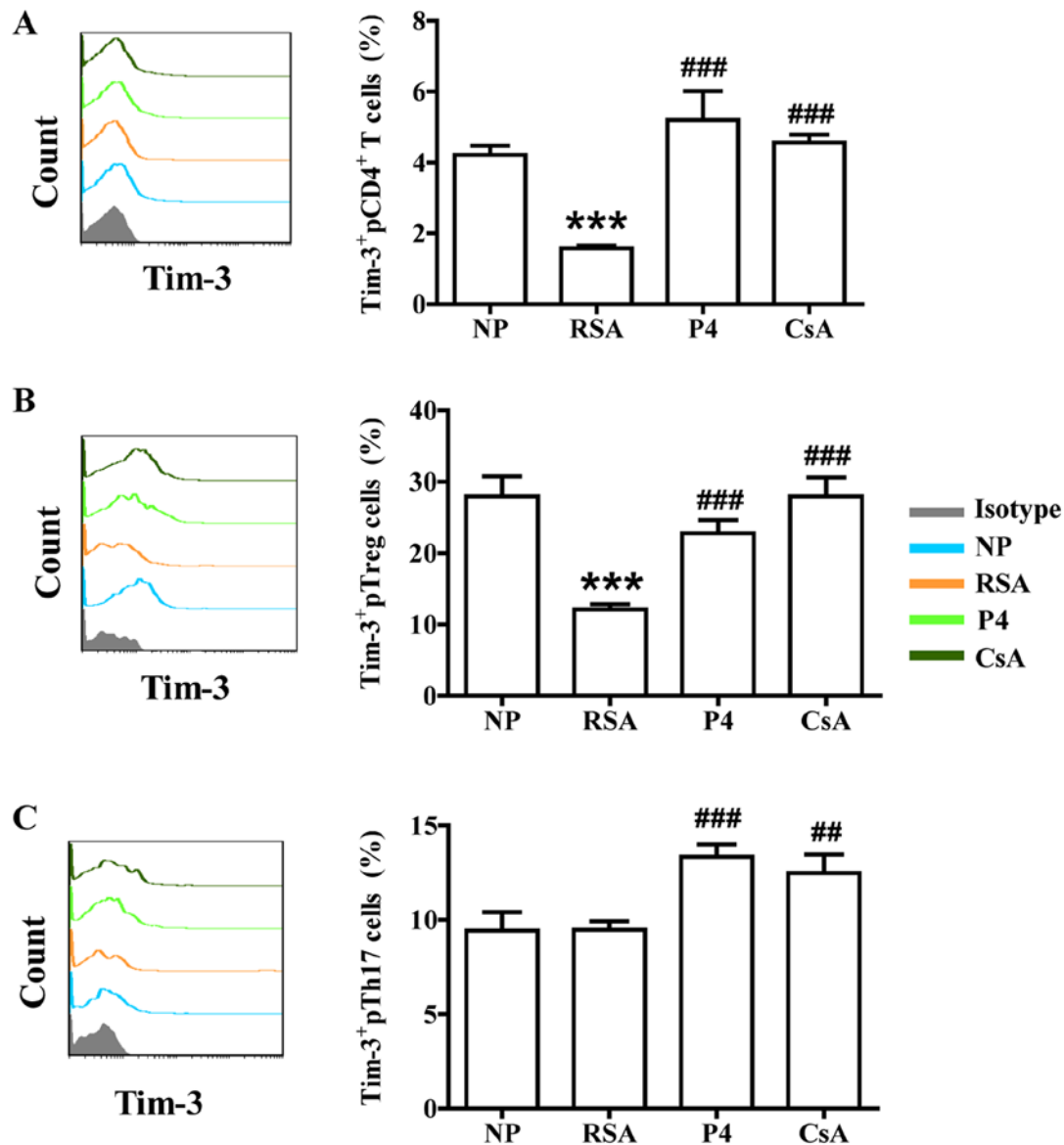


Figure 5. Effects of P4 and CsA on Tim-3 expression in peripheral blood cells of pregnant females with a history of RSA. Compared with that in the NP group, the production of Tim-3 in (A) CD4⁺ T cells and (B) Treg cells in the peripheral blood of subjects in the RSA group was decreased. (C) In Th17 cells in the peripheral blood, no significant difference in Tim-3 expression between the RSA and NP group was determined. P4 and CsA promoted Tim-3 expression in all three indicated subsets of T cells. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. ***P<0.001 vs. NP; **P<0.01, ###P<0.001 vs. RSA. CsA, cyclosporine A; pTreg cells, peripheral T-regulatory cells; Th17 cells, type-17 T-helper cells; RSA, recurrent spontaneous abortion; P4, progesterone; TIM-3, T-cell immunoglobulin mucin family member-3.

iii) improved adhesion and invasion of mouse preimplantation embryos (44); and iv) promoted trophoblastic invasion and proliferation at low dosages (0.0001-1 μ mol/l) (45,46). Small-scale clinical observations (47,48), in addition to unpublished data by our group, have indicated that CsA significantly increased the live birth rate in cases of RSA without increasing complications during pregnancy and among neonates.

The present study suggested that CsA reduced the proportion of Th17 cells and promoted Treg-cell dominance among pregnant females with a history of RSA. CsA decreased the production of IL-17A (the major effector cytokine of Th17 cells) and also upregulated the expression of TGF- β 1, IL-10, CTLA-4 and Tim-3. These results suggest that CsA, as a trophoblast function promoter, also restores

the Th17/Treg-cell balance, while inducing Th2 bias during pregnancy. Accordingly, future research group will be aimed at studying the effect of P4 and CsA on other pro-inflammatory cytokines in Th17 cells, including IL-6, IL-21 and IL-23. Further investigations are required to explore whether non-pathogenic Th17 subsets and pro-inflammatory Th17 subpopulations co-exist at the maternal-fetal interface, and which mechanisms may be used to modulate Th17 subsets during pregnancy.

To date, RSA with its underlying causes and mechanisms has remained to be fully elucidated. Studies have indicated that the more abortions occur, the higher the likelihood of abortion during the next pregnancy. For instance, after two spontaneous abortions, the risk of spontaneous abortion during the third pregnancy is ~30%, whereas after >3

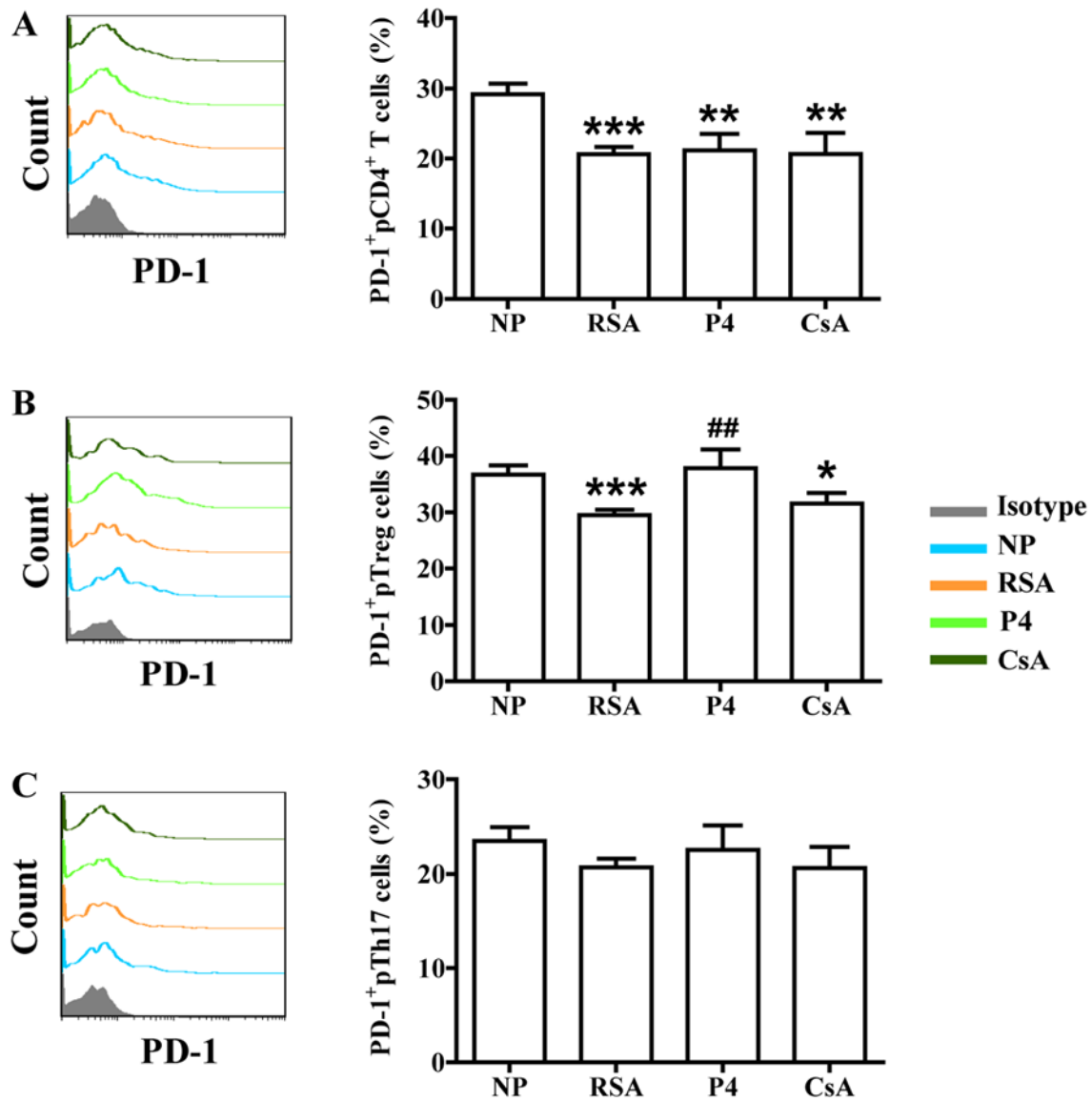


Figure 6. Effects of P4 and CsA on PD-1 expression in peripheral blood cells of pregnant females with a history of RSA. Compared with that in the NP group, the production of PD-1 in (A) CD4⁺ T cells and (B) Treg cells in the peripheral blood of subjects in the RSA group was decreased. (C) In Th17 cells in the peripheral blood, no significant difference in PD-1 expression between the RSA and NP group was determined. While P4 and CsA had no effects on PD-1 expression in total CD4⁺ T cells and Th17 cells, P4 promoted PD-1 expression in Treg cells. Groups: NP, normal early pregnancy; RSA; patients with RSA; P4, pregnant females with a history of RSA treated with P4; CsA, pregnant females with a history of RSA treated with CsA. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. NP; ##*P*<0.01 vs. RSA. CsA, cyclosporine A; pTreg cells, peripheral T-regulatory cells; Th17 cells, type-17 T-helper cells; RSA, recurrent spontaneous abortion; P4, progesterone; PD-1, programmed cell death-1.

spontaneous abortions, the risk of spontaneous abortion during the next pregnancy maybe as high as 45% (49). Early diagnosis and treatment of RSA has been of great significance to families. Although previous studies have focused on the maternal-fetal interface, non-invasive examination is of great importance in clinical practice. The present study investigated the imbalance between Th17 and Treg cells in the peripheral blood to improve the current understanding of the pathogenesis of RSA. The results confirmed that the percentage of Treg cells and the expression of TGF- β 1, IL-10, CTLA-4 and Tim-3 decreased in the peripheral blood of RSA patients, which may provide an experimental basis for non-invasive monitoring indicators of pregnancy. However, while the experiments were performed using flow cytometry with PMA stimulation, determining the serum

levels of cytokines in peripheral blood or cytokine production in different T-cell subpopulations through ELISA or PCR may have been more representative of the *in vivo* situation. Of course, further follow-up and larger-scale studies are required to confirm whether such molecules maybe used as monitoring indicators of pregnancy. The present results revealed that, while CsA reduced the proportion of Th17 cells and promoted Treg-cell dominance, P4 may also have an immunomodulatory role through certain co-inhibitory molecules to exert its treatment effect in RSA. However, the small sample size and lack of follow-up of pregnancy outcomes have limited the assessment of the effects of P4 and CSA on fetal protection in the present study. However, follow up of the subjects of the present study regarding pregnancy outcomes will continue and, in the future, the sample size will be expanded.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SCW and MDL carried out the experiments. SCW, DJL and MRD conceived experiments and analyzed data. MDL, CQC, FRS, JFY and JFQ coordinated the sample collection, data interpretation, literature search, and figure preparation. JFQ and SCW drafted the manuscript. MRD revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval of the present study was obtained from the ethics committee of the Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China) and all subjects provided written informed consent.

Patient consent for publication

All subjects provided written informed consent for the publication of associated data.

Competing interests

The authors declare that they have no competing interests.

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