



Research article

CPY27B1-related vitamin D deficiency contributes to the impaired function of CD4⁺T cells in Recurrent spontaneous miscarriage

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ABSTRACT

The study aimed to explore the relationship between the expression of cytochrome P450 family 27 subfamily B member 1 (CYP27B1), vitamin D, and impaired T cell subsets in recurrent spontaneous miscarriage (RSM). A Total of 779 healthy women of childbearing age and 1031 women with a history of RSM were involved in this study. The results of flow cytometry showed that the proportion of Tregs was higher in healthy women than in the women with RSM. For cytokines, the levels of interleukin-17 (IL-17) and interferon-gamma (IFN- γ) were significantly higher in RSM patients than in healthy women, while IL-10 was notably lower in RSM patients. Furthermore, compared to healthy individuals, RSM patients had lower levels of serum 25(OH)D detected by chemiluminescence. The frequency of Tregs was negatively correlated with 25(OH)D. Specifically, for every 10 ng/ml increase in 25(OH)D, the percentage of Tregs increased by 0.58 as calculated. IL-17 and IFN- γ were inversely correlated with 25(OH)D, while the serum interleukin-10 (IL-10) level was positively correlated with 25(OH)D. CYP27B1 was found to be expressed in both cytotrophoblast and extracellular villi trophoblast cells. However, reduced expression of CYP27B1 was observed in the placenta with RSM. Notably, the level of 25(OH)D increased in the supernatant of CYP27B1 knockdown BeWo compared to normal cells, while human chorionic gonadotropin (hCG) was significantly reduced. The hCG secretion of CYP27B1 KO BeWo cells was partially restored after 1,25(OH)₂D₃ supplementation. In addition, 1,25(OH)₂D₃ treatment could induce more CD4⁺ T cells to convert to Foxp3⁺Treg, which in turn inhibited the secretion of IL-17, IFN- γ . In summary, this research unveiled a connection between reduced CYP27B1 and vitamin D deficiency in RSM. Our study underscores the potential benefits of vitamin D treatment supplementation in the context of RSM. However, it is important to note that further research is imperative to validate these observations.

1. Introduction

Recurrent spontaneous miscarriage (RSM), also known as recurrent spontaneous abortion (RSA), is a condition where a woman experiences two or more consecutive miscarriages before the 20th week of gestation. The causes of miscarriage are complex and

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multifactorial, involving genetic, immunological, hormonal, and environmental factors related to the pregnant woman, the fetus, or the placenta. However, up to 50 % of the causes of miscarriages in women of childbearing age remain unknown. As successful pregnancies rely on balanced immune responses, immunological aberrancy was reported to contribute up to 50 % of poor pregnancy outcomes [1].

T cells are a type of white blood cell that play a crucial role in the immune system. They are responsible for recognizing and attacking foreign substances, such as viruses and bacteria, as well as the developing fetus. Several studies have investigated the potential role of T cells in the development of RSM [2,3]. One theory suggests that an imbalance in the levels of different T cell subsets, such as T helper (Th)1 and Th2 cells [4], may contribute to the development of RSM. Other studies have explored the role of regulatory T cells (Tregs) in the development of RSM [5].

The potential role of vitamin D in RSM has been investigated. Several studies and meta-analysis found that women with RSM had significantly lower vitamin D levels compared to women without RSM [6–8]. Vitamin D deficiency was suggested to be a risk factor for RSM. A study investigated the association between vitamin D levels and immune cells in women with RSM. The results showed that women with RSM had lower vitamin D levels and higher levels of pro-inflammatory T cells than women without RSM, suggesting that vitamin D supplementation may improve the chances of a successful pregnancy via a balance of immune cells [9]. For vitamin D₃ metabolism, it depended on cytochrome enzymes CYP27B1 in the kidney [10]. In the meantime, extrarenal production of 1,25(OH)₂D₃, likely for paracrine or autocrine uses, was recognized widely in many tissues, including the epidermis and other epithelial tissues, placenta, bone, and tumors [11]. A few observational studies showed reduced expressions of CYP27B1 in chorionic villi and decidua compared with normal pregnant women [12,13]. However, there were no significant differences in the localization of CYP27B1 and cytokines IL-10, IFN- γ , tumor necrosis factor- α (TNF- α) between the normal pregnant and miscarriage women.

Epidemiological and experimental data suggested that vitamin D might exert its biological functions in the placenta associated with CYP27B1 and T cell subsets in miscarriage [13,14]. Here, we aim to figure out whether the decreased expression of CYP27B1 in the placenta may contribute to reduced active vitamin D levels in RSM pregnancies and may be involved in impaired T cell subsets in the Chinese RSM. Furthermore, 1,25(OH)₂D₃ supplement helps to restore the dysfunction of CD4⁺T cell, allowing us to gain a better understanding of the mechanism of action and the potential benefits of vitamin D supplementation in women with RSM who have decreased CYP27B1 expression.

2. Materials and methods

2.1. Subjects

This was a case-control study. The participants were recruited from the outpatient department at the Center for Reproductive Medicine, Obstetrics & Gynecology Hospital of Fudan University (Shanghai, China) from January 2018 to October 2022. Excluding anatomic, endocrine, or genetic factors, women with a history of three or more consecutive spontaneous miscarriage were diagnosed as RSM. Women of childbearing age without RSM history, cancers, autoimmune disease, severe infectious diseases etc., defined as healthy donors (HDs). Controls were randomly selected with respect to age. The exclusion criteria were patients with acute or chronic infection, autoimmune disease, history of cardiovascular disease, history of endocrine disease, recent use of drugs that affect the immune response, significant liver and kidney dysfunction, and malignant tumors. Peripheral blood was taken during the first prenatal visit, and lymphocyte subset analysis were performed in 779 healthy women of childbearing age and 1031 women with a history of habitual spontaneous miscarriage (≥ 3) determined by ultrasound. For serum vitamin D and cytokines testing, 73 healthy women of childbearing age and 30 women with a history of habitual spontaneous miscarriage (≥ 3) were collected. Placental tissues from 3 miscarriage women with a history of habitual spontaneous miscarriage and 3 non-disease women who had experienced induced abortions were performed by immunofluorescence analysis and Western blot.

The human choriocarcinoma trophoblast cell line BeWo was purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). All patients in this study were anonymous, following the principles of the Declaration of Helsinki and approved by Ethics Committee of the Obstetrics & Gynecology Hospital of Fudan University.

2.2. Blood sampling and biomarker measurement

Blood samples used for this study were collected at the time of admission to the Obstetrics department. We used Abbott's architect Alinity Immunoassay Analyzer and 25-OH Vitamin D Reagent Kit to measure total 25(OH)D in serum. The assay is a chemiluminescent microparticle immunoassay in which peripheral blood is centrifuged at 3000 \times g for 10 min, 1 h after the blood is drawn, and the type of specimen used for the 25(OH)D assay is serum. Cytokines IL-10, IL-17, TNF- α / β , IFN- γ were instantly measured by flow cytometry in the laboratory of Obstetrics and Gynecology Hospital of Fudan University. Sera for further measurements were collected and frozen at -80°C until used.

2.3. Flow cytometric analysis

Peripheral blood lymphocytes (PBL) in the 2 ml heparinized blood was freshly isolated by Ficoll density gradient centrifugation. Lymphocytes were re-suspended in PBS supplemented with 2 % bovine serum albumin (BSA) at a concentration of 1×10^6 cells/ml. Cell surface marker analysis was performed using four or five color flow cytometric analysis. Cyto-STAT tetraCHROME CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 Antibody Cocktail (Beckman Coulter), IOTest CD19-PC5 (Beckman Coulter), CD3-FITC/CD(16 + 56)-

PE (Beckman Coulter), IOTest CD45-PC7 (Beckman Coulter), IOTest CD3-FITC (Beckman Coulter), IOTest CD4-PC5 (Beckman Coulter), IOTest CD25-PE (Beckman Coulter), IOTest CD127-APC (Beckman Coulter), CD3 eFlour 450 (UCHT1; eBioscience, CA, USA), CD4-PC5 (13B8.2; Beckman Coulter, CA), IL-17A-PE (BL168; Biolegend), CD25-APC (BC96; eBioscience), Foxp3-Ax488 (PCH101; eBioscience), and the secondary antibody were used, together with appropriate isotype controls, to allow identification of positive and negative cell populations. Flow cytometry instantly measured the Cytokines IL-10, IL-17, TNF- α/β , and IFN- γ (Kuang Bo Tong Sheng Biotechnology Co., Ltd, Tianjin, China). For double staining of IL-17 and Foxp3 (Forkhead box protein p3), PBL were isolated using Ficoll density gradient separation, stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) (Sigma-Aldrich), and operated using the Cytotfix/Cytoperm kit from BD Biosciences (San Diego, CA, USA) according to the instructions. Multiple-color FACS analysis was performed using a BD FACSCanto II flow cytometer (BD). Approximately 1×10^4 to 1×10^5 cells were analyzed, and the gating strategy for the identification of T subsets was performed as described previously [15] (Supporting information, Fig. S1a).

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNeasy Kits (Qiagen, USA) according to the manufacturer's instructions. The concentration and quality of the total RNA were assessed with a NanoDrop spectrophotometer (Thermo, USA). Then first-strand cDNA was subsequently synthesized using PrimeScript RT Kit (Takara, Japan) according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed in triplicate on a 7900 HT real-time PCR system (Applied Biosystems, USA) using SYBR Premix Ex Taq (TaKaRa, Japan). The results were analyzed using the $2^{-\Delta\Delta ct}$ calculation method and GAPDH was used as an endogenous control [15]. The primers used for the experiments are listed in Supplementary Table 1.

2.5. In vitro cell culture and cell line

2.5.1. CD4⁺T cells cultivation

For cell sorting, 10 ml of peripheral blood was collected from either HDs or patients, and CD4⁺T cells were obtained from PBL using the human CD4⁺T Cell Isolation Kit II (Miltenyi Biotec). The purity of the cells was 90 % or greater as determined by re-analysis. Typically, the cells were incubated with 1,25(OH)₂D₃ (Sigma-Aldrich) at a final concentration of 20 nM or DMSO as control under the stimulation with precoated 5 μ g/ml α CD3, soluble 5 μ g/ml α CD28 and 200U/ml rhIL-2 at 1×10^5 per well in 96-well U-bottom plates, and three replicate wells were set up. The cells were cultured in a humidified CO₂-containing atmosphere at 37 °C for 5–7 days in a complete RPMI-10 medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.5 mM sodium pyruvate, 0.05 mM nonessential amino acids, 2 mM L-glutamine, and 10 mM HEPES (all from GIBCO). For cell proliferation, purified T cell was stained with CFSE and detected cell division by flow cytometry after 5 days of incubation [15].

2.5.2. BeWo choriocarcinoma cells cultivation and cell transfection

BeWo choriocarcinoma cells were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and were maintained as monolayers in Kaighn's modification of Ham's F-12 medium (Sigma Aldrich, St. Louis, USA) supplemented with 10 % fetal bovine serum (FBS) under standard culture conditions of 5 % CO₂ in air at 37 °C with medium renewal every 2–3 day. CYP27B1 knockdown as well as BeWo cell culture supernatants after 1,25(OH)₂D₃ induction were collected for 25(OH)D and hCG analysis. The shRNA technique was used to knock down the expression of CYP27B1 in BeWo cells. CYP27B1 shRNA target sequence was GGTC AAGGAAGTGCTAAGA. CYP27B1 shRNA plasmid and control plasmid were from Shanghai Qihe Biotechnology Co., Ltd. (China). BeWo were seeded in 6-well culture plates and subsequently infected with CYP27B1 shRNA plasmid or control plasmid at the exponential phase for 48 h, using Lipofectamine[™] 3000 Transfection Reagent (L3000015, Thermo Fisher, USA). Next, fresh complete medium was substituted for further cultivation. The infection efficiency was evaluated by Western blot 48 h after infection [16].

2.6. Western blot

Cells were treated with RIPA Lysis Buffer (Beyotime, P0013B) containing 1 % phenylmethanesulfonyl fluoride (PMSF) for protein extraction. While for placental tissues, the lysis process was assisted with tissue homogenizers (Miltenyi). BCA protein assay kit (Beyotime, P0012) was applied to quantify protein concentration. The lysates were then boiled (10 min, 95 °C) for denaturation. Protein (25 μ g) was loaded on 10 % SDS-PAGE gel with markers and electrophoresed by using a Miniprotein III system (Bio-Rad, 1658033). Afterward, it was transferred to PVDF membranes (Millipore, ISEQ00010) for 90 min and immersed in 5 % skimmed milk for 1 h at room temperature to block non-specific binding. The PVDF membranes were incubated with primary antibodies against CYP27B1 (1:500, ab206655, abcam), β -catenin (1:500, Abcam, ab230169) or GAPDH (1:1000, ab75479, abcam) overnight at 4 °C respectively. Then PVDF membranes were washed with PBST solution three times and treated with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000; Bioworld Technology, BS10003) or goat anti-mouse IgG secondary antibody (1:5000; Bioworld Technology, BS10003) at room temperature for 1 h. After adequate washing, Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore, WBKLS100) was used for chemiluminescence detection [16].

2.7. Immunofluorescence staining

For immunofluorescence analysis, formalin-fixed, paraffin-embedded clinical tissue sections were prepared and stained using human CYP27B1, human leukocyte antigen-G (HLA-G), and hCG antibodies and a VECTASTAIN Elite ABC kit (Vector Laboratories)

according to the manufacturer’s protocol. Rabbit anti-human CYP27B1 monoclonal antibodies (1:500, ab206655, Abcam), mouse anti-human CYP27B1 monoclonal antibodies (1:500, sc-515903, SANTA CRUZ), Mouse anti-human HLA-G monoclonal antibodies (1:50, 4H84, SANTA CRUZ), Rabbit anti-human hCG monoclonal antibodies (1:1000, ab238319, Abcam) were used as the primary antibody. HRP conjugated Goat Anti-Rabbit IgG (1:500, Servicebio), 488 conjugated Goat Anti-Rabbit IgG (1:400, Servicebio), Cy3 conjugated Goat Anti-mouse IgG (1:300, Servicebio) were used as the secondary antibody, all slides were evaluated by two pathologists.

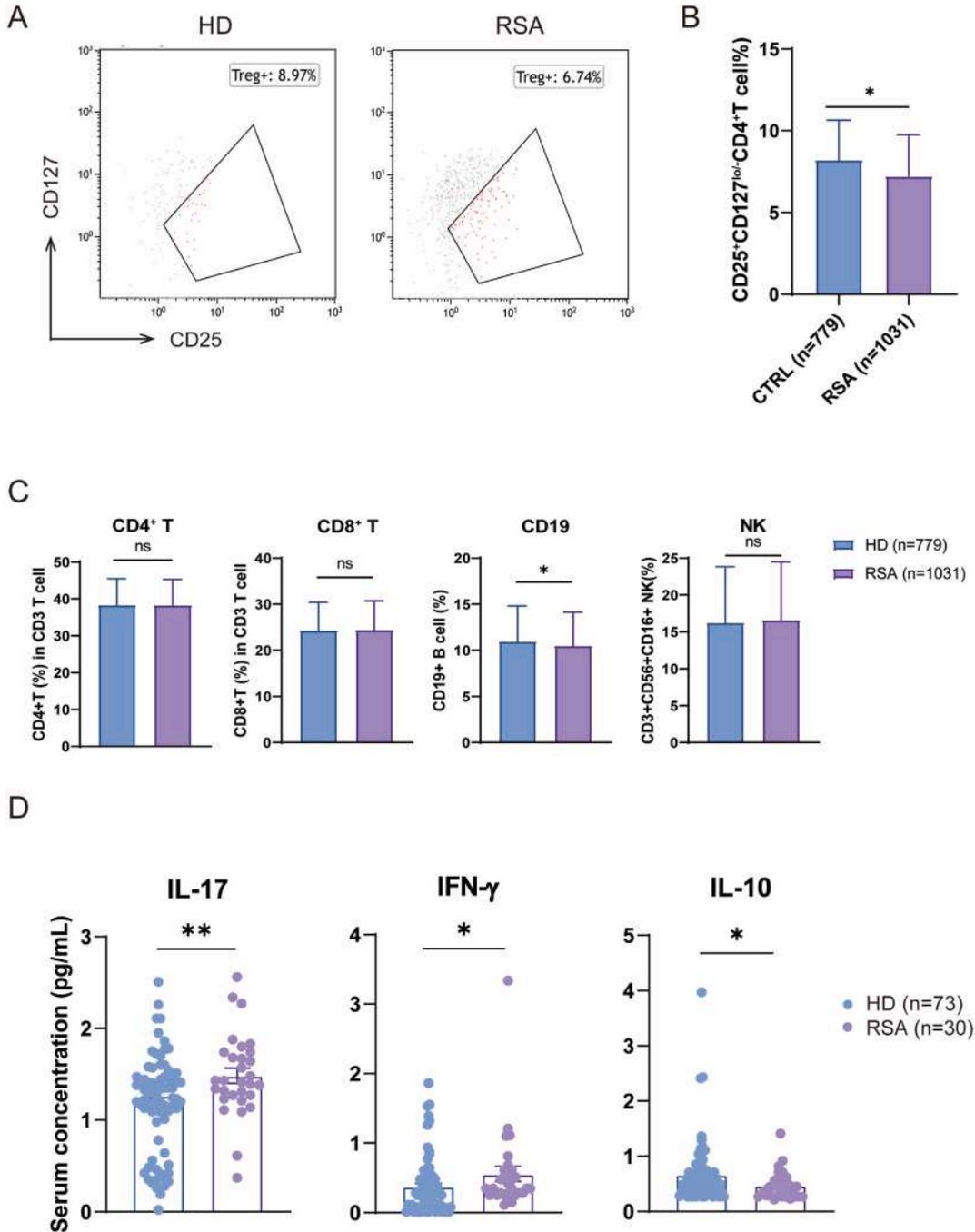


Fig. 1. The circulating T cell subsets and cytokines in patients with RSM. (A) Representative flow cytometric dot plots of peripheral CD25⁺CD127^{lo/-}CD4⁺ T cell in HD (left) and RSM (right). (B) Bar plot of CD25⁺CD127^{lo/-}CD4⁺ T cell in RSM and HD. (C) The frequencies of CD4, CD8, B cell, and NK cell in RSM patients compared to HD. (D) Serum concentrations of IL-17, IL-10, and IFN-γ in RSM and HD. Data represent the mean ± SEM. Statistical differences were determined by a two-tailed unpaired Student *t*-test. **p* < 0.05, ***p* < 0.01 as compared with HD.

2.8. Statistics

The statistical evaluation was performed with GraphPad Prism (version 8.0; GraphPad Software, CA). Values are shown throughout the manuscript as mean \pm SEM except for the patients and HD age, which is shown as mean \pm SD. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student *t*-test for normally distributed data. In the case of significant differences between subgroups, post hoc analyses were based on the Tukey test (normally distributed data) and on Friedman nonparametric test (abnormally distributed data). Pearson's correlation coefficient (normally distributed data), and Spearman's rank correlation coefficient (abnormally distributed data) were used to assess interrelationships. A smoothed curve fit plot was created to examine the shape of the relationship between serum vitamin D and the ratio of peripheral blood Treg cells. Logistic regression models were used to examine the effect of serum vitamin D on the ratio of peripheral blood Treg cells. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Decreased treg cells and elevated serum IFN- γ , IL-17 levels in patients with RSM

To investigate whether Treg and Th17 cells played a role in the development of RAS, we measured the number of circulating Treg and Th17 in PBL using flow cytometry. We defined the phenotype of Treg cells as CD4⁺CD25⁺CD127^{lo} (Supplementary Fig. 1). As shown in Fig. 1A and B, the proportion of Tregs in HDs ranged from 5.8% to 10.6%, compared to 4.7%–9.6% in women with RSM ($p < 0.05$). There were no significant changes in the frequencies of CD3 subsets. In HDs, the proportion of CD4⁺ and CD8⁺ T cells in the total CD3⁺ T cells ranged from 31.2% to 45.5% and 31.4%–45.3%, while in patients with RSM, these percentages ranged from 18.2% to 30.4% and 18.2%–30.5%, respectively (Fig. 1C). The frequencies of NK cells, defined as CD3⁺CD56⁺CD16⁺, remained unchanged in RSM compared to HDs (16.61% \pm 7.90% vs 16.24% \pm 7.61%, $p = 0.67$) (Fig. 1C). CD19⁺ B cells were significantly lower in RSM compared to HDs (10.50% \pm 3.64% vs 10.95% \pm 3.86%, $p < 0.05$) (Fig. 1C). Overall, the data showed the main change in peripheral blood cell subsets in RSM patients was a decrease in Treg cells.

Next, the circulating cytokines were investigated by Luminex assay. IL-17 and IFN- γ as pro-inflammatory cytokines increased significantly in RSM than HDs (1.44 \pm 0.51 vs 1.28 \pm 0.81 pg/ml, 0.54 \pm 0.59 vs 0.40 \pm 0.44 pg/ml, $p < 0.05$). IL-10 as inflammatory cytokines decreased significantly in RSM than HDs (0.46 \pm 0.26 vs 0.65 \pm 0.56 pg/ml, $p < 0.05$) (Fig. 1D). While there were equivalent concentrations of TNF- α and β observed in RSM and HD controls (Supplementary Fig. 2A).

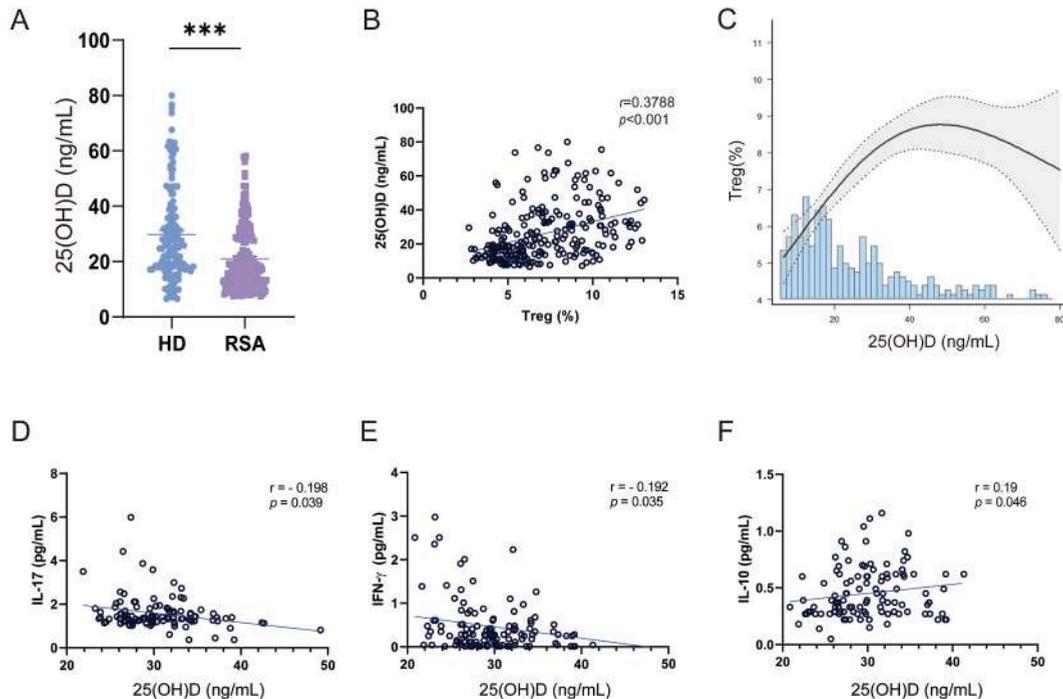


Fig. 2. The correlation between 25(OH)D and Treg or related cytokines. (A) Dot plot of serum concentrations of 25(OH)D in RSM (N = 121) and HD (N = 121). (B) Linear regression was applied to analyze the correlation between 25(OH)D level and Treg. (C) The blue histogram represents the distribution of serum vitamin D. The black solid line represents the fitting curve of serum vitamin D and peripheral blood Treg, and the black curve represents the 95% confidence interval of the curve. (D–F) Correlations between serum 25(OH)D level and serum IL-17, IL-10 and IFN- γ . Data represent the mean \pm SEM. Statistical differences were determined by a two-tailed unpaired Student *t*-test. *** $p < 0.001$ as compared with HD.

3.2. The relationship between serum vitamin D and treg frequencies, along with pro- and anti-inflammatory cytokines

There is a growing body of evidence indicating an association between vitamin D deficiency and T cell subsets in the context of RSM [17]. To assess whether the serum 25(OH)D is linked to Treg levels and associated cytokines, we initially measured the concentration of serum 25(OH)D and Treg both in RSM patients and healthy donors. In comparison to HDs, the RSM patients exhibited lower levels of 25(OH)D (Fig. 2A). We also observed a negative correlation between the frequency of Treg and 25(OH)D ($r = 0.3788$, $p < 0.001$) (Fig. 2B). Smoothed curve fit plots illustrated a dose-response relationship between serum vitamin D levels and peripheral blood Tregs (Fig. 2C). Specifically, for every 1 ng/ml increase in 25(OH)D, the Treg percentage increased by 0.06. A similar pattern was observed, with a 0.58 increase in Treg percentage for every 10 ng/ml rise in vitamin D, as calculated (Table .1).

Additionally, we found that pro-inflammatory cytokines such as IL-17 and IFN- γ , associated with Th17 and Th1 subsets, displayed an inverse correlation with 25(OH)D ($r = -0.198$, $p = 0.039$ and $r = -0.192$, $p = 0.035$, respectively) (Fig. 2D and E). On the other hand, serum IL-10 levels exhibited a positive correlation with 25(OH)D ($r = 0.197$, $p = 0.046$) (Fig. 2F). Nevertheless, there was no significant correlation between TNF- β and 25(OH)D ($r = 0.2296$, $p = 0.1125$) (Supplementary Fig. 2B). However, a positive correlation was noted between TNF- α with 25(OH)D ($r = 0.2938$, $p = 0.0405$) (Supplementary Fig. 2B). Although vitamin D was indicated to play an important role in immune-related RSM, the relationship between circulating 25(OH)D and these factors did not consistently align. We postulated that there might be additional influencing factors, such as CYP27B1.

3.3. Reduced expression of CYP27B1 in patients with RSM

Extrarenal expression of the enzyme CYP27B1, which was responsible for generating 1,25(OH) $_2$ D $_3$, has been discovered in many tissues. We applied an immunohistochemistry assay and Western blot to assess the expression and localization of CYP27B1. In normal placental tissue, we found an average nTPM of CYP27B1 at 0.3 (Fig. 3A) (<https://www.proteinatlas.org/ENSG00000111012-CYP27B1/tissue#rna%20expression>) [18]. We observed CYP27B1 (Green) and the somatic marker hCG (Red) were co-located in the normal placenta tissue, while cell nuclei were stained with DAPI (Blue). The findings suggested that cytotrophoblasts expressed CYP27B1 (Fig. 3B). In addition, we found CYP27B1 expressed in extracellular villus trophoblast cells (EVT) as well when using HLA-G (Red) as a marker of EVT in the villus (Fig. 3C). Further analysis showed that RSM placental tissue exhibited reduced levels of CYP27B1 expression in comparison to placental tissue from normal pregnancies, particularly at the protein level (Fig. 3D, Supplementary Fig. 6).

3.4. Down-regulated CYP27B1 in BeWo contributed to low 1,25(OH) $_2$ D $_3$ level

BeWo, a cell line derived from a placenta with choriocarcinoma, possessing an epithelial morphology, demonstrated high CYP27B1 expression. To down-regulate CYP27B1 expression in BeWo, we applied shRNA for long-term knockdown (Fig. 4A, Supplementary Fig. 7). Trophoblast cells might hydroxylate 25(OH)D into 1,25(OH) $_2$ D $_3$ via CYP27B1. Since 1,25(OH) $_2$ D $_3$ had a short half-life, 25(OH)D was commonly used for detection. Simultaneously, we assessed hCG, a glycoprotein secreted by placental trophoblast cell, to evaluate its function. 25(OH)D significantly increased in the supernatant of CYP27B1 KO BeWo compared to normal cells, along with a significant reduction in hCG ($p < 0.05$) (Fig. 4B and C). The hCG secretion in CYP27B1 KO BeWo cells was partially restored after supplementation with 1,25(OH) $_2$ D $_3$ (Fig. 4C). These findings collectively indicated that CYP27B1 related vitamin D deficiency occurred in the placenta.

3.5. 1,25(OH) $_2$ D $_3$ inhibits IL-17A, IFN- γ and promotes Foxp3 expression in CD4 $^+$ T cells in RSM patients

To assess the effects of vitamin D $_3$ supplementation on T cell, T cells from HD cultured with α CD3/CD28 and subjected to a series of concentrations of 1,25(OH) $_2$ D $_3$ treatment for 5 days. 1,25(OH) $_2$ D $_3$ treated cell yielded similar apoptosis compared to DMSO (Supplementary Fig. 3). Similarly, median dose 1,25(OH) $_2$ D $_3$ treatment did not affect the apoptosis of CD4 T cells in RSM (Fig. 5A and B). FOXP3 and Retinoic acid receptor-related orphan receptor gamma t (ROR γ t) were key transcription factors for Treg and Th17 cells, respectively. Treatment with median dose 1,25(OH) $_2$ D $_3$ significantly upregulated FOXP3 and decreased the ROR γ t expression in CD4 $^+$ T cells (Fig. 5C). Furthermore, we isolated CD4 $^+$ T cell from RSM patients to investigate the 1,25(OH) $_2$ D $_3$ effect on T cell subsets. The results showed that the percentage of IL-17, IFN- γ and a portion of TNF- α secreting CD4 $^+$ T reduced after 1,25(OH) $_2$ D $_3$ treatment for 3 days in a dose dependent manner ($p < 0.05$) (Fig. 6A–C). CD4 $^+$ T cells were initially purified from HD and exhibited similar results (Supplementary Fig. 4). There was a relatively high level of IFN- γ and TNF- α double positive CD4 $^+$ T cell after 1,25(OH) $_2$ D $_3$ treatment in RSM compared to HD. Additionally, there was lower IL-17 and ROR γ t mRNA, along with higher Foxp3 mRNA expression higher

Table 1

Logistic regression analysis of the proportion of serum vitamin D to peripheral Treg cells in RSM.

Exposure	Non-adjusted		Adjust model	
	OR (95 % CI)	p value	OR (95 % CI)	p value
Vitamin D (ng/mL)	0.06 (0.04, 0.08)	<0.0001	0.06 (0.04, 0.08)	<0.0001
Vitamin D per 10 ng/ml	0.58 (0.40, 0.77)	<0.0001	0.58 (0.40, 0.76)	<0.0001

Adjust I model adjust for Age.

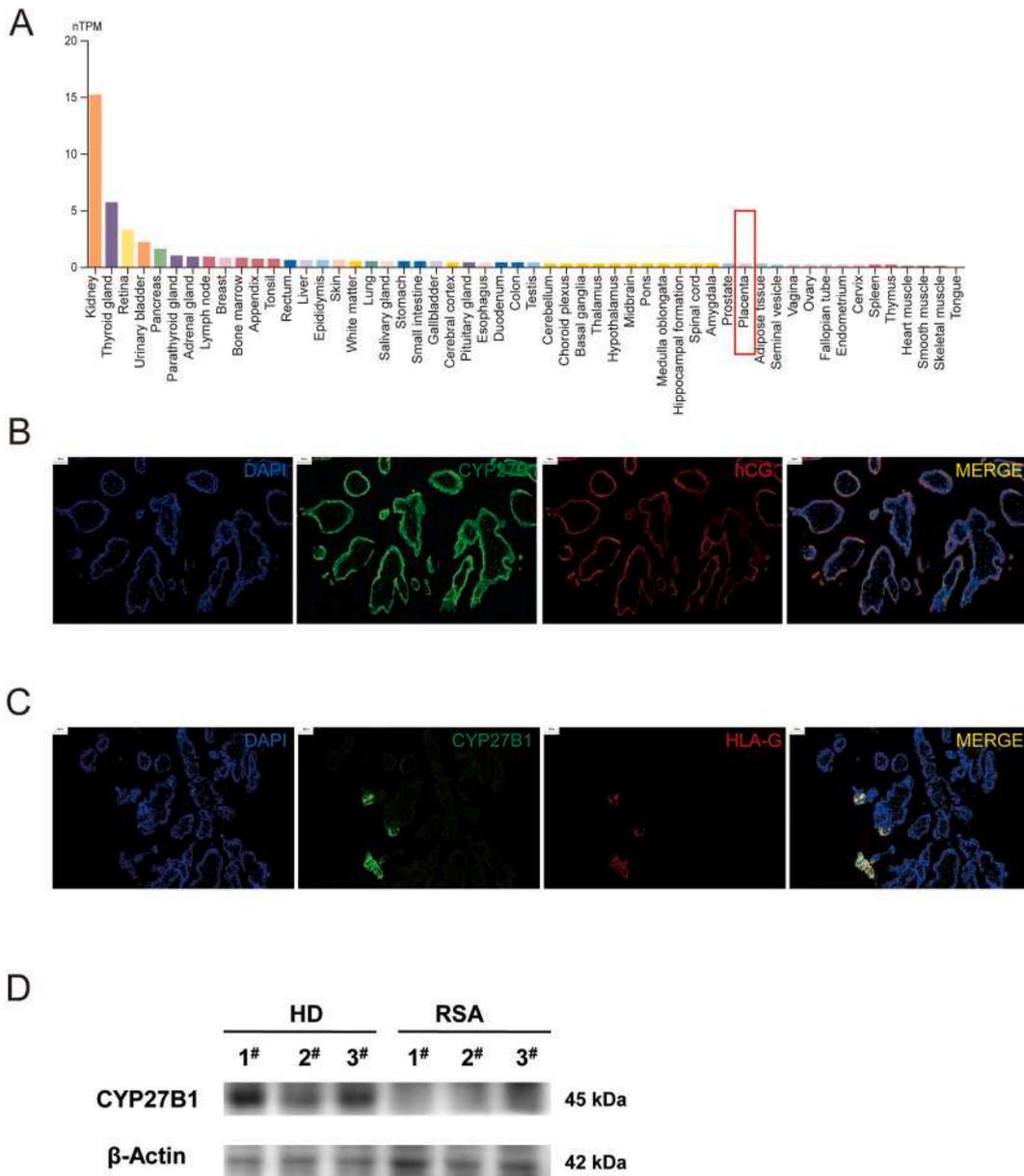


Fig. 3. Placental CYP27B1 protein expression in normal and RSM tissue. (A) CYP27B1 expression in human tissues shown here is in part based upon data generated by The Human Protein Atlas: version 21.1. (proteinatlas.org). (B) Detection of CYP27B1 expression in frozen placenta tissue by immunofluorescence staining. The representative images of cytotrophoblasts by confocal microscopy showed CYP27B1 (green) and hCG (red) expression. (C) Representative images of extracellular villus trophoblast cells showed CYP27B1 (green) and HLA-G (red) expression in frozen tissue. Nuclei were stained with DAPI (blue). (D) CYP27B1 expression in the placenta of RSM and HD was measured by WB. The full image can be found in [Supplementary Fig. 6](#).

Foxp3 mRNA expression in CD4⁺ T cells ([Fig. 6D](#) and [Supplementary Fig. 5](#)). In summary, the results showed that 1,25(OH)₂D₃ treatment induced a greater conversion of CD4⁺ T cells to convert to Foxp3⁺iTreg, while inhibited the secretion of IL-17, IFN-γ and part of TNF-α.

4. Discussion

Vitamin D has been gaining increased attention in recent years regarding its potential role in reproductive physiology [1]. However, there is still a lack of clinical data to definitively establish a clear connection between vitamin D deficiency with recurrent pregnancy loss [9,19,20]. In this study, we observed a correlation between circulating 25(OH)D₃ and T cell subsets, along with related

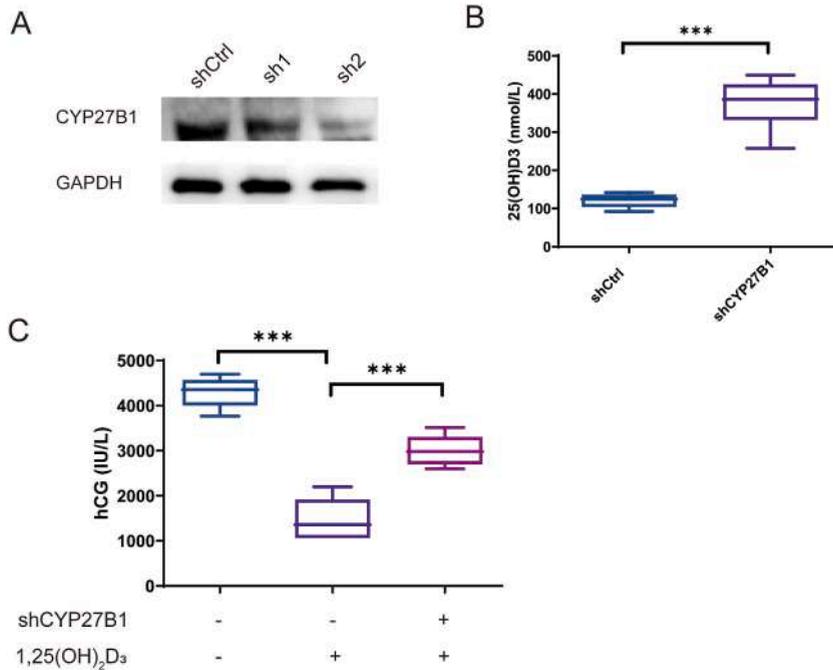


Fig. 4. The knockdown of CYP27B1 in BeWo and its effect on hCG secretion. (A) The knockdown efficiency of the shRNA targeting CYP27B1 was confirmed by Western blot. The full image can be found in [Supplementary Fig. 7\(B\)](#) Bar plot of 25(OH)D level detected by immunoassay in the supernatant of shCYP27B1 (N = 6) and shCtrl (N = 6) BeWo. (C) Bar plot of hCG secretion of shCYP27B1 BeWo with or without 1,25(OH)₂D₃ treatment (N = 6). Data represent the mean ± SEM. Statistical differences were determined by a two-tailed paired Student *t*-test. ****p* < 0.001 as compared with shCtrl.

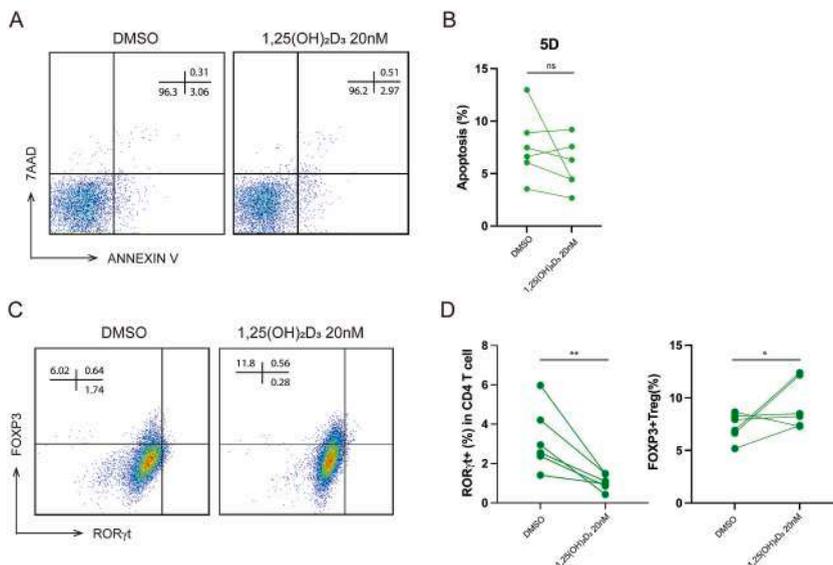


Fig. 5. The impact of 1,25(OH)₂D₃ on CD4⁺T cell in RSM. (A) CD4⁺ T cells purified from RSM were activated by αCD3/28 in the present of 20 nM 1,25(OH)₂D₃ or DMSO for 5 days. The collected cells were then analyzed for apoptosis by FACS. Representative flow cytometric dot plots of apoptosis are shown. (B) The statistical analyses were conducted using data from six samples. (C) Representative flow cytometric dot plots of FOXP3 and RORγt expression on day 5. (D) Statistical analysis of the percentage of FOXP3 (right) and RORγt (left) in CD4⁺T were presented. Statistical differences were determined by a two-tailed paired Student *t*-test. **p* < 0.05, ***p* < 0.01 as compared with DMSO treatment.

cytokines, such as IFN-γ, TNF-α, and IL-17. We assumed that a decreased expression of CYP27B1 in villous and decidual tissues may contribute to low VD levels, particularly in terms of the local production of 1,25(OH)₂D₃ at the fetal-maternal interface. The supplementation of 1,25(OH)₂D₃ was able to restore the balance of T cell subsets, pro- and anti-inflammation cytokines in RSM.

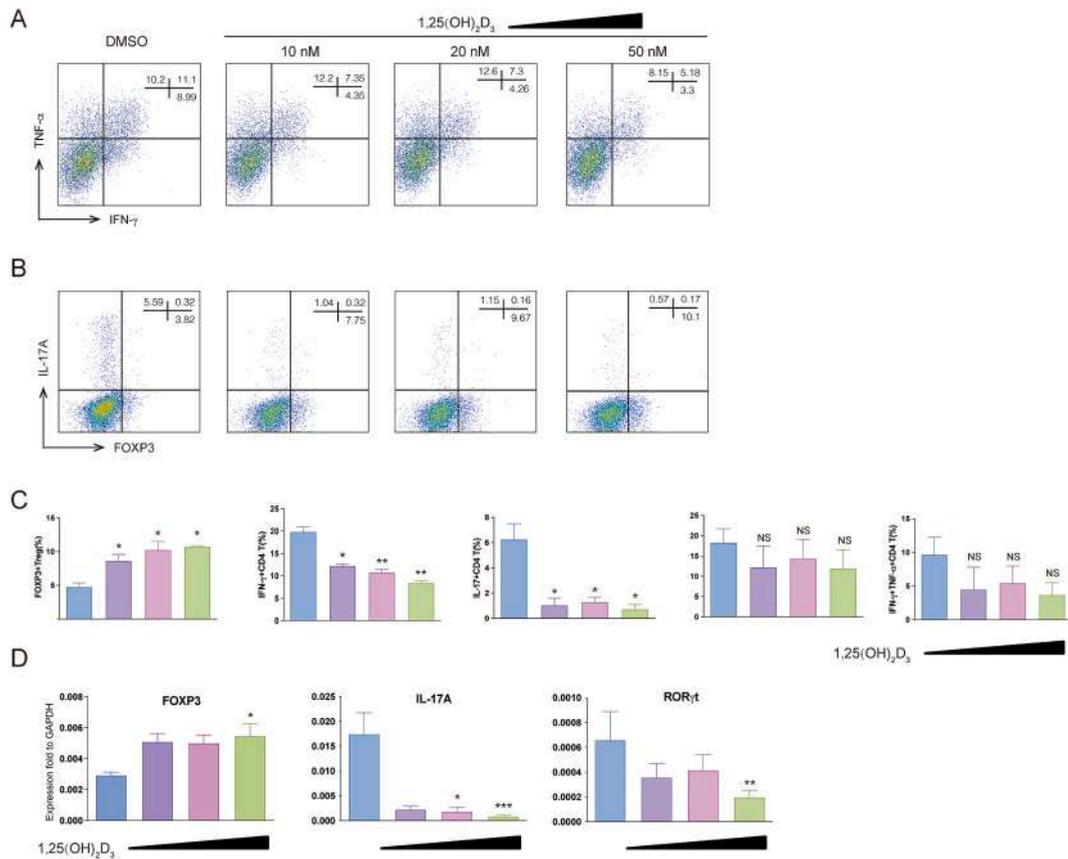


Fig. 6. 1,25(OH)₂D₃ treatment rescued the aberrant expressions of cytokines in CD4⁺ T cells. CD4⁺ T cells purified from RSM were activated by αCD3/28 in a series concentration of 1,25(OH)₂D₃ or DMSO for 5 days. The collected cells were then analyzed for apoptosis (Annexin V⁺/7AAD⁻ cells) and necrosis (Annexin V⁻/7AAD⁺ cells) by FACS. (A) Representative flow cytometric dot plots of IFN-γ and TNF-α double staining in CD4⁺ T cell after 5 days of culture with 1,25(OH)₂D₃ or DMSO. (B) Representative flow cytometric dot plots of Foxp3 and IL-17 double staining in CD4⁺ T cell after 5 days of culture with 1,25(OH)₂D₃ or DMSO. (C) Bar plot showed the frequencies of Foxp3⁺Treg, IFN-γ⁺CD4⁺T, TNF-α⁺CD4⁺T, IL-17⁺CD4⁺T, and IFN-γ⁺TNF-α⁺CD4⁺T cell. (D) The mRNA expression of FOXP3, IL-17A, and RORγt was measured by real-time PCR. Data represent the mean ± SEM, N = 4. Statistical differences were determined by friedman nonparametric test. **p* < 0.05, ***p* < 0.01 as compared with DMSO treatment (Blue bar).

Several risk factors have been found to contribute to recurrent pregnancy loss, including structural abnormalities of the uterus and autoimmune disorders. The reasons why some pregnancies were affected while others were not remained unclear [1]. Whereas innate and adaptive immune matters in a successful pregnancy in which T cells played a significant role. Based on the different CD markers, T cells were basically categorized into three groups: CD4⁺ Th cells, CD8⁺ T cytotoxic (Tc) cells, and CD4⁺CD25⁺ T regulatory cells. Ota et al. reported a higher percentage of B cells and NK cells in women with RSM, with no significant difference in the percentage of T cells [6]. In this study, which included relatively large population of women with RSM (n = 1031) and controls (n = 779), we found no significant differences in the levels of Th and Tc cells between the RSM and control groups. Similarly, there were no significant changes on NK cell as well. However, it is worth noting that our study did not focus on the NK cytotoxicity, and further investigation is warranted. Interestingly, we observed a decreased B lymphocytes in cases of RSM, which was often associated with antiphospholipid syndrome, a known cause of RSM.

Inflammation is a major factor influencing pregnancy outcomes [3,21]. A large body of evidence has demonstrated that an excess of pro-inflammatory cytokines such as IL-17, IFN-γ and TNF-α, can lead to reduced fetal viability [22,23]. Vitamin D was also reported to inhibit the proliferation of Th1 and limit their production of these cytokines. Similarly, in CD4⁺T cell from HD, an additional treatment with 1,25(OH)₂D₃ inhibited IL-17 and IFN-γ remarkably. As for TNF-α alone, 1,25(OH)₂D₃ treatment partially reduced its level, suggesting that excess TNF-α may be secreted by other cells, such as macrophages. Interestingly, there was no significant difference in circulating TNF-α/β between RSM and HD, which needed explored in different cell types. Flow cytometry results indicated a population of IFN-γ and TNF-α double positive CD4⁺T cell after αCD3/28 stimulation. Following 1,25(OH)₂D₃ treatment, this population could decrease by two-thirds in HD, but by less than half in RSM. Of several cytokines, only synergism of TNF-α and IFN-γ was reported to induce PANoptosis [24]. Further studies are required to help us understand whether TNF-α and IFN-γ together induced pyroptosis is involved in the progress of RSM.

It is importance to understand vitamin D physiology because a high proportion of women with RSM were diagnosed with VD

insufficiency (VDI) or VD deficiency (VDD) following clinical guidelines based on observational studies [6]. Vitamin D can be obtained from both endogenous sources, such as exposure to ultraviolet light, and exogenous, including certain foods, and dietary supplements. It underwent a 2-step sequential hydroxylation (25-hydroxylase and 1- α -hydroxylase) to produce an active metabolite, 1,25(OH)₂D₃ also known as calciferol. Downstream vitamin D functioned by binding to and activating the nuclear vitamin D receptor (VDR). When activated by its ligand, VDR preferentially forms heterodimers with RXR to bind specific DNA sequences known as vitamin D response elements (VDREs). In the absence of a ligand, it can also form complexes with corepressors, such as NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) and histone deacetylases to mediate gene transcription [25]. Recently, an alternative pathway of vitamin D metabolism involving cytochrome P450scc (CYP11A1) was reported [26]. CYP11A1 can hydroxylate the side chain of vitamin D₃ at carbons 17, 20, 22 and 23 to produce at least 10 metabolites [27]. These metabolites could be detected in human epidermis and serum and had the ability to influence the expression of differentiation and antioxidant genes in keratinocytes through interaction with VDR. This recent discovery highlighted the complexity of vitamin D metabolism [28] and extended our understanding of the diverse pathways of vitamin D metabolism in the body, providing new avenues for further research into the biological functions of vitamin D in RSM.

There are relatively few studies concerning VD levels at fetal-maternal interface, as well as the expression and activity of related molecules involved in VD metabolism. Some of these studies had yielded different results and were based on small sample sizes. One group suggested RSM and normal women had the same quantity and function of VDR, especially in endometrial cells [29,30]. In contrast, the VDR expression showed no significant difference between women with RSM and a control group (Data not shown). In addition to VDR, vitamin D and its derivatives can act via ROR α and ROR γ as well [31,32]. In cancer, the expression of the vitamin D metabolite receptor ROR α/γ is negatively correlated with melanoma progression and is associated with a favorable prognosis [26]. However, there was limited report of ROR α/γ on trophoblasts in RSM, which requires further investigations.

It had been reported that the expression and localization of CYP27B1 at the fetal-maternal interface [12]. Here, we found that both cytotrophoblasts and extracellular villus trophoblast cells expressed CYP27B1. In RSM's placenta, CYP27B1 expression is significantly downregulated, which may contribute to the aberrant VD metabolism and function at the fetal-maternal interface. We applied shRNA to knock down CYP27B1 expression in BeWo, and results showed that 1,25(OH)₂D₃ treatment could rescue its hCG secretion. The result supported that the local expression of CYP27B1 might maintain a high level of 1,25(OH)₂D₃ at the fetal-maternal interface.

One study found a crude live birth rate associated with serum vitamin D was seen in women undergoing assisted reproductive treatments, but statistical significance was lost once important prognostic factors were taken into account. Meanwhile, it is not recommended that women try to conceive and take vitamin D supplements in the absence of vitamin D deficiency [33]. In the field of reproduction, observational, prospective, and retrospective studies robustly advocated for a positive role of vitamin D. While the most substantial randomized controlled trial (RCT) employing a sizable sample size and high-dose supplementation failed to validate these observations [34]. Considering the aberrant vitamin D metabolism at the fetal-maternal interface, exogenous intake of vitamin D may not be fully utilized locally. In our study we showed a pronounced downregulation of CYP27B1 in placenta of RSM. Local reduced 1,25(OH)₂D₃ appeared to be closely associated with impaired activation of vitamin D pathways in the placenta. It is worth considering whether vaginal administration of vitamin D, and/or in combination with progesterone [35] could have a beneficial impact on preventing spontaneous miscarriage, such as luteal-phase support during in vitro fertilization (IVF). Besides, our findings provided insights into one potential factor contributing to the etiology of this condition. Indeed, it is crucial to recognize that that unexplained habitual miscarriage may be underpinned by a multitude of pathogenic mechanisms linked to abnormal immune function, and these mechanisms can vary among individuals.

Our findings offered an alternative perspective on the complex web of pathogenic mechanisms of RSM. Nonetheless, it is essential to acknowledge certain limitations that warrant consideration. Firstly, despite the exclusion of patients with autoimmune diseases from our study cohort, there remains the possibility that some cases of unexplained habitual miscarriage were not accounted for. Patients with autoimmune disorders might present alternative immune-related mechanisms that have yet to be elucidated. Consequently, our study solely elucidates a segment of the underlying causes. Secondly, our research did not specifically investigate the activation of the nonclassical vitamin D pathway (CYP11A1-mediated) at the placental interface. Moreover, considering the high prevalence of vitamin D deficiency (<30 nmol/L) and inadequacy (<50 nmol/L) was found in Chinese adults as reported [36]. It's important to note that our experimental results we observed were based on the Asian population. We did not directly observe the relationship between circulating vitamin D and local vitamin D level in the placenta. Indeed, for a more comprehensive understanding of these relationships, further research and experimental evidence need to be conducted in a more diverse range of populations, including participants from different races, geographical backgrounds, and genetic characteristics.

Overall, our study suggested a potential link between CYP27B1-related vitamin D deficiency, altered T cell subsets, and cytokines imbalances in RSM. Acknowledging the potential presence of aberrant vitamin D metabolites in the placenta, our results lend support to the idea that local vitamin D supplementation could offer potential benefits. Nevertheless, it is crucial to emphasize that further experimental and observation research are needed to validate these conclusions and establish the ideal dosage and duration of vitamin D supplementation for women with RSM.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

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Ethics approval statement and consent statement

All patients in this study were anonymous, following the principles of the Declaration of Helsinki and approved by Ethics Committee of the Obstetrics & Gynecology Hospital of Fudan University (2019-06). The data are anonymous, and the requirement for informed consent was therefore waived.

CRediT authorship contribution statement

Chaoyan Yue: Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yanhui Ma:** Writing - original draft, Methodology, Investigation, Data curation. **Mingyan Wang:** Methodology, Investigation. **Minmin Yuan:** Methodology, Investigation. **Yi Meng:** Methodology, Investigation. **Zhiheng Wang:** Methodology. **Chunmei Ying:** Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e24499>.

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