Article

Discovery Medicine 2025; 37(193): 276–285
https://doi.org/10.24976/Discov.Med.202537193.22

Silencing TGF-β3 Alleviates Recurrent Spontaneous Abortion Inflammation in Mice: the Importance of Treg/Th17 Cell Balance

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Published: 20 February 2025

Background: Recurrent spontaneous abortion (RSA), also known as repeated miscarriage, refers to the consecutive loss of pregnancy three times or more before the fetus reaches viability. In this study, we aimed to investigate the impact of transforming growth factor β3 (TGF-β3), which plays a crucial role in immune dysregulation, on the imbalance of regulatory T cell (Treg) and T helper 17 (Th17) cells.

Methods: First, we isolated T cells from an RSA mouse model we established in-house. Tregs and Th17 cells were labeled by targeting forkhead box protein P3 (Foxp3) and retinoic-acid-receptor-γt (RORγt), respectively, and the levels of Tregs and Th17 were determined by flow cytometry. The expression levels of Foxp3, RORγt, TGF-β3, interleukin (IL)-10, and IL-17 in T cells were measured by means of reverse-transcription quantitative polymerase chain reaction (qRT-PCR) and Western blotting. The levels of interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4 in mouse serum were quantified using enzyme-linked immunosorbent assay (ELISA). si-TGF-β3 was transfected into RSA mice, and the expression levels of IL-17 and CD25 were determined by flow cytometry, during which T cells were labeled with antibodies against IL-17 and CD25, respectively. Additionally, si-TGF-β3 or TGF-β3 interference therapy was administered to RSA mice, and the expression levels of IL-1β, tumor necrosis factor-α (TNF-α), IL-6, IFN-γ, GM-CSF, and IL-4 in mouse serum were measured using ELISA.

Results: In the RSA model, there was a significant decrease in the percentage of Treg cells, alongside an elevation of *TGF-β3* mRNA ($p < 0.05$). The percentage of Th17 cells in RSA mice significantly increased and correlated positively with TGF-β3 levels. In RSA, the levels of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IFN-γ, and GM-CSF increased, while those of anti-inflammatory cytokine IL-4 decreased ($p < 0.05$). Transfection of si-TGF-β3 into RSA mice reduced the percentage of Th17 cells and increased the percentage of Tregs and Treg/Th17 ($p < 0.05$). Increased levels of Th17-related markers and reduced levels of Tregs-related markers occurred following the administration of TGF-β3 to RSA mice ($p < 0.05$). Transfection of si-TGF-β3 into RSA mice also resulted in a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines ($p < 0.05$), while TGF-β3 administration reversed these changes in RSA mice, indicating the role of TGF-β3 in modulating the inflammatory response during RSA.

Conclusions: Knockdown of TGF-β3 enhanced Treg/Th17 balance in RSA, suggesting TGF-β3 as a potential therapeutic target for RSA.

Keywords: Treg cells; Th17 cells; TGF-β3; recurrent spontaneous abortion

Introduction

Recurrent spontaneous abortion (RSA) is a complex, multifactorial pathological process mediated by a variety of cell types at the maternal-fetal interface [1–3]. RSA occurs in 15% to 25% of all clinical pregnancies, and the incidence could be extrapolated to 57% if preclinical losses are taken into account [4,5]. Common risk factors for RSA include advanced maternal age, fetal chromosomal abnormalities, alcohol usage, smoking, and so on [5,6]. Immunological mechanisms, particularly those involving alterations in maternal immune tolerance towards the fetus, have gained considerable attention in RSA research [7]. Dysregulation of regulatory T cell (Treg) and T helper 17 (Th17) cell populations, along with aberrant cytokine profiles, contributes to maternal-fetal immune tolerance breakdown, leading to recurrent pregnancy loss [8]. Other well-known pathogenic factors include infections such as syphilis, as well as chronic diseases such as diabetes and autoimmune disorders. Nevertheless, the etiology of more than half of RSA patients remains unknown [7].

Immunological tolerance between mother and fetus built upon by immune T cells is essential for the establishment and maintenance of normal pregnancy, which is comparable to an effective semi-allotransplantation. T cells have a significant regulatory role within this immune net-

work. The pro-inflammatory or anti-inflammatory character of certain cell subsets is a key factor in fetal destiny determination [9]. Central to the inflammatory process are $CD4^+$ T cells, which include Th1 cells, Th2 cells, Treg cells, and Th17 cells [10]. Th2-dominant immunity was previously thought to be linked to a successful pregnancy, and a Th1/Th2 cell imbalance is regarded as the contributing factor to unplanned abortion [11]. In particular, it has been postulated that the outcomes of early and late pregnancy are reliant on the equilibrium between Treg cells and Th17 cells [12,13]. Tregs are crucial for maintaining maternal-fetal immune tolerance by suppressing immune responses against the fetus [14]. Conversely, Th17 cells, known for their pro-inflammatory properties, are implicated in inflammatory processes and autoimmune conditions. The balance between Tregs and Th17 cells is essential for successful pregnancy outcomes, and disruptions in this balance have been associated with pregnancy complications, including RSA [15].

The opposite effects on inflammation are experienced by Tregs and Th17 cells, although having the identical origin. The main functions of Tregs are to preserve immunological self-tolerance and prevent autoimmune illness [16], thereby contributing to hampering autoimmune disease development as well as anticancer responses [17,18]. Tregs exhibit the forkhead box protein P3 (Foxp3) and produce interleukin (IL)-10, IL-35, and transforming growth factor beta (TGF-β), which contribute to their immunosuppressive functions [19]. The success of pregnancies has also been shown to depend on Th17 cells, which take part in inflammatory responses [10]. In order to effectively stimulate retinoic-acid-receptor-γt (RORγt) as well as IL-17 production, the Th17 differentiation needs pro-inflammatory cytokines such as IL-6 and TGF-β [20].

TGF-β is a pleotropic cytokine involved in many biological processes. *In vivo* research has been performed relating transforming growth factor β3 (TGF-β3), one of the three TGF isomers, to development [21], but the implication of TGF-β3 in the immune system has rarely been reported. However, recent evidence from a murine study advocates the significance of TGF-β3 in influencing the immune system. For example, the characteristics of Th17 cells produced in the presence of TGF-β3 differ from those produced in the presence of TGF-β1 [22]. In addition, TGF-β3 is also generated by $CD4^+$ $CD25^-$ $LAG3^+$ Tregs, and early growth response protein-2 and lymphocyte-activation gene 3 (LAG3) proteins are markers of Tregs that produce IL-10 [23,24]. TGF-β3 has garnered attention for its regulatory role in maternal-fetal immune tolerance. It has been shown to promote Treg differentiation and function while inhibiting Th17 cell differentiation and pro-inflammatory cytokine production [25]. A prior study has indicated that dysregulation of TGF-β3 signaling is implicated in various pregnancy disorders, including RSA [26]. Thus, targeting TGF-β3 signaling pathways presents a promising approach for mitigating immune dysregulation and improving pregnancy outcomes in RSA.

Recent research has shown that maintaining a balance between Tregs and Th17 cells is essential for ensuring a healthy pregnancy, while RSA, pre-eclampsia, and gestational diabetes mellitus have all been linked to alterations in the Treg/Th17 ratio in favor of Th17 cells [27–29]. Despite advances in understanding the immunological mechanisms underlying RSA, there remains a gap in elucidating the precise role of TGF-β3 in modulating Treg/Th17 cell balance and its therapeutic potential in RSA management. Therefore, the objectives of this study are to investigate the effects of TGF-β3 silencing on Treg/Th17 cell balance and inflammatory responses in a mouse model of RSA. Elucidation of the molecular pathways could provide insights into novel therapeutic strategies for mitigating inflammation and preventing recurrent pregnancy loss.

Materials and Methods

Enzyme-Linked Immunosorbent Assay (ELISA)

The expression levels of these cytokines in serum were measured with IL-1β (ml098416), TNF-α (ml002095), IL-6 (ML063159), IFN-γ (ml002277), GMCSF (ml037645) and IL-4 (ml064310) ELISA kits. All ELISA kits were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). OD values were analyzed by enzyme-labeled instrument (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA).

Western Blotting

Western blotting was performed in accordance with standard procedures. Cells (1×10^5 cells/mL) were treated as directed, washed with PBS, and then lysed with RIPA buffer (Thermo Fisher Scientific, 87787, Waltham, MA, USA) supplemented with a cocktail of protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific, 78420, Waltham, MA, USA). After centrifugation at 12,000 $\times g$ for 15 min at 4 °C, the protein concentration of the lysates was assessed using a BCA assay (Beyotime, P0010, Shanghai, China). The proteins were then electrophoresed via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–12% gel. The separated protein bands were subsequently transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, GVWP02500, Darmstadt, Germany). After blocking with 5% skim milk for 2 h, the primary antibody was to the membrane for incubation at 4 °C overnight. The primary antibodies utilized in this experiment include TGF-β3 (1:1000; Sigma-Aldrich, SAB2103852, St. Louis, MO, USA), Foxp3 antibody (1:1000; Sigma-Aldrich, ABE75, St. Louis, MO, USA), RORγt (1:1000; Thermo Fisher Scientific, PA5-23148, Waltham, MA, USA), and glyceraldehyde

3-phosphate dehydrogenase (GAPDH) (1:1000, Sigma-Aldrich, G5262, St. Louis, MO, USA). ECL Kit (BioRad, 171-3064, Helsinger, CA, USA) was used for chemiluminescence signal detection after incubating the membrane with horseradish peroxidase-labeled secondary antibodies (1:1000, ab6721, Abcam, Cambridge, MA, USA).

Isolation and Purity of Naïve CD4$^+$ T Cells

Subsets of T cells were extracted from the spleen and para-aortic lymph nodes of mice under normal pregnancy or RSA mice. Mouse CD4$^+$ T Cell Isolation Kit (B90001, Selleck, Shanghai, China) was used to isolate mouse CD4$^+$ naive T cells. Every procedure was completed in a sterile setting and in compliance with the manufacturer's guidelines. According to flow cytometric results, over 93% of naïve CD4$^+$ T cells were pure in isolated cell suspensions. The mycoplasma detection results for all cell cultures tested in this study were negative. All cells involved in this study have undergone morphological identification and surface marker identification. For the identification results, please refer to **Supplementary Fig. 1** (The purity of naïve CD4$^+$ T cells was 98.6%).

Cell Culture

The cells used in this study were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultures were maintained in a 37 °C, 5% CO_2 humidified incubator. The medium was periodically refreshed to ensure a continuous nutrient supply for robust cell growth. Passaging was performed when cell confluence reached 80% to prevent overgrowth.

Cell Transfection

Both sets of small interfering RNA, si-TGF-β3 and negative control (si-NC) were acquired from GenePharma (Shanghai, China). si-NC: 5′-TTCTCCGAACGTGTCACGT-3′; si-TGF-β3: 5′-CGGUGCUUGGACUAUACAATT-3′. CD4$^+$ T cells isolated from RSA mice were transfected with si-TGF-β3. Transfection of si-TGF-β3 and si-NC was performed according to the manufacturer's instructions. The transfection mixture containing siRNA and transfection reagent was added to the cultured CD4$^+$ T cells, followed by an incubation period to ensure efficient uptake of siRNA by the cells.

Total RNA Extraction and RT-PCR Assays

Using an RT-PCR reverse transcription Kit (KR103, TIANGEN, Beijing, China), cDNA was produced from 1 µg of total RNA. Using the One Step qRT-PCR Kit (Invitrogen, 11736051, Carlsbad, CA, USA) in accordance with the manufacturer's instructions, reverse-transcription quantitative polymerase chain reaction (qRT-PCR) was carried out to detect the expression of TGF-β3, Foxp3, RORγt, IL-10, IL-17, and GAPDH. The changes of ploidy at the gene level were measured by the $2^{-\Delta\Delta CT}$ method, with GAPDH as the normalization gene. The sequences of the primers used in this experiment are as follows:

TGF-β3, F: 5′-CCTGGCCCTGCTGAACTTG-3′, R: 5′-TTGATGTGGCCGAAGTCCAAC-3′; Foxp3, F: 5′-CCCTGACCAAGGCTTCATCTG-3′, R: 5′-GGAGCAACTCTGGGAATGTG-3′; RORγt, F: 5′-CTGGAAGTGGTGCTGGTT-3′, R: 5′-CGGAGAAGTCAAAGATGGAG-3′; IL-10, F: 5′-GGAGAACCTGAAGACCCT-3′, R: 5′-TGATGAAGATGTCAAACTCACT-3′; IL-17, F: 5′-TGTCACTGCTACTGCTGCT-3′, R: 5′-GGTTATGGATGTTCAGGTTG-3′; GAPDH, F: 5′-GGACCTGACCTGCCGTCTAG-3′, R: 5′-GTAGCCCAGGATGCCCTTGA-3′.

Animal Experiments

Male and female mice with the designations CBA/J and DBA/2 were acquired from Wuhan Yunclone Technology Co., Ltd. in China (Certificate No. SN0174553). The mice were domesticated in an environment with controlled humidity (50 ± 5%) and temperature (25 ± 2 °C), and given food and water access *ad libitum*. We found that crossbreeding the CBA/J (female) DBA/2 mice (male) is more likely to create progenies that are RSA-inducible. The lentiviral transfection complex (si-NC or si-TGF-β3) was transfected into CD4$^+$ T cells extracted from the mouse tissues. The viral library was transduced into mice via intraperitoneal injection. The mating of CBA/J (male) and DBA/2 mice (female) also produced an animal model of RSA [30]. Only female and male mice aged 10 weeks, which had reached sexual maturity, were selected for mating; the mating procedure was conducted by confining a male mouse and a female mouse in a cage. In this experiment, we regarded the presence of vaginal suppositories or sperm on vaginal smears the next morning after the supposed mating as the first day of pregnancy during the observation period of 60 days. After 60 days of gestation, blood was collected, followed by intraperitoneal injection of pentobarbital sodium (3 mg/mL) (110 mg/kg) for euthanasia, and hysterectomy. The serum was then extracted and stored at –20 °C for further use. Obtain mouse placental tissue for relevant experiments. This study has been approved by the Experimental Animal Ethics Committee of the First Hospital of Hunan University of Chinese Medicine (Approval No: ZYFY-20230319-003).

Flow Cytometry

After performing cell lysis, the lysate (R0010, Solarbio, Beijing, China) was mixed with an appropriate concentration of fluorescently labeled antibodies, such as CD4 (1:1000, ab133616, Abcam, Cambridge, MA, USA) and CD25 (1:1000, ab314083, Abcam, Cambridge, MA, USA). The mixture was incubated at room temperature for 30 minutes to allow the antibodies to bind to the corresponding antigens on the cell surface.

Next, the resulting samples were fixed and permeabilized as necessary according to experimental requirements to allow the detection of intracellular markers. An appropriate concentration of fluorescently labeled antibodies, such as Foxp3 (1:1000, ab20034, Abcam, Cambridge, MA, USA), RORγt (1:1000, ab280197, Abcam, Cambridge, MA, USA), IL-17 (1:1000, ab125029, Abcam, Cambridge, MA, USA), and IL-25 (1:1000, ab180594, Abcam, Cambridge, MA, USA) was then added to the fixed and permeabilized lysate. The mixture was incubated at 4 °C for 1 hour to facilitate binding of the antibodies to their respective intracellular antigens. After incubation, the lysate was washed to remove unbound antibodies. The samples were subsequently analyzed using a flow cytometer (CytoFLEX, Beckman Coulter, Fullerton, CA, USA) [31].

Statistical Analyses

The results are expressed as mean ± standard deviation. GraphPad Prism software version 8.0 (GraphPad Inc., San Diego, CA, USA, https://www.graphpad-prism.cn/) was employed for statistical analyses. Multiple group comparisons were conducted using ANOVA, followed by Bonferroni's post hoc test, with $\alpha = 0.05$. Comparisons between two groups were analyzed using an independent sample *t*-test. Spearman's correlation analysis was performed to examine the association between Treg/Th17 and TGF-β3.

Results

TGF-β3 was Increased while the Treg/Th17 Ratio was Decreased in the RSA Mouse Model

The Th17 master regulator RORγt can be directly influenced by Foxp3 and become antagonistic in action [32–34]. In RSA, the percentage of Tregs became dramatically lower ($p < 0.05$) (Fig. 1A,C), along with a significant increase in *TGF-β3* mRNA levels ($p < 0.05$, R = –0.698; Fig. 1E). Meanwhile, the percentage of Th17 cells was significantly increased in RSA ($p < 0.05$) (Fig. 1B,C), positively correlating with TGF-β3 levels ($p < 0.05$, R = 0.6138; Fig. 1E). Additionally, according to the results of Western blotting and qRT-PCR, compared to the normal pregnancy (NP) group, the mRNA and protein levels of TGF-β3 and RORγt were significantly elevated in the RSA group, while the mRNA and protein levels of Foxp3 were significantly decreased ($p < 0.05$) (Fig. 1D,E). Moreover, the results shown in Fig. 1F,G demonstrate that the ratio of Foxp3/RORγt mRNA and protein levels was significantly reduced in the RSA group ($p < 0.05$) (Fig. 1F,G). RSA mice also had a noticeably reduced Treg/Th17 ratio ($p < 0.05$), and TGF-β3 levels were adversely linked with this ratio ($p < 0.05$, R = –0.6968; Fig. 1F,G).

Inflammatory Response was Augmented in Mouse Model of RSA

Fig. 2 demonstrates that compared to the NP group, the RSA group experienced an increase in levels of pro-inflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α), IL-6, interferon-γ (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) and a reduction in the level of IL-4, an anti-inflammatory cytokine ($p < 0.05$), collectively pointing to the augmented inflammatory response in RSA.

TGF-β3 Silencing Restored the Normal Treg/Th17 Ratio in RSA Mouse Model

TGF-β3 was downregulated in the placenta of si-TGF-β3-transfected RSA mice, as compared to si-NC-transfected RSA mice (Fig. 3A; $p < 0.05$), indicating the successful silencing of TGF-β3. Additionally, the $CD4^+$ $CD25^+$ $CD127^-$ cells were classified as Tregs, and the $CD4^+$ $IL\text{-}17^+$ cells were categorized as Th17 cells. We found that the Th17 percentage was decreased (Fig. 3B,C; $p < 0.05$) while the Tregs percentage and Treg/Th17 ratio were increased (Fig. 3D–F; $p < 0.05$) in si-TGF-β3-transfected RSA mice compared to their si-NC-transfected counterparts. Considering that the TGF-β3 level was increased on top of the changes to the above-mentioned parameters in RSA, we concluded that the TGF-β3 positively regulated Th17 while negatively regulated Tregs and Treg/Th17 in RSA, correcting the imbalance in the Treg/Th17 ratio. To further understand the regulatory role of TGF-β3 in Tregs and Th17 cells, we administered TGF-β3 to RSA mice, and consequently, the mice exhibited increased expression of Th17-related RORγt and IL-17 and reduced expression of Treg-related Foxp3 and IL-10, as compared to RSA mice that were not given the same TGF-β3 treatment (Fig. 3G; $p < 0.05$). These results proved that the decreased Treg/Th17 ratio was related to the increased TGF-β3, and mechanistically speaking, TGF-β3 silencing could restore the normal Treg/Th17 ratio.

TGF-β3 Silencing Suppressed Inflammatory Response in RSA Mouse Model

Fig. 4 demonstrates that the levels of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, IFN-γ, and GM-CSF were decreased while the level of anti-inflammatory cytokine IL-4 was increased in si-TGF-β3-transfected RSA group compared to their si-NC-transfected RSA group ($p < 0.05$). On the contrary, the levels of IL-1β, TNF-α, IL-6, IFN-γ, and GM-CSF were increased while the level of IL-4 was decreased in TGF-β3-treated RSA group compared to RSA mice that were not treated with TGF-β3 ($p < 0.05$). These results indicated that during RSA, increased TGF-β3 was involved in the augmentation of inflammatory response while silencing TGF-β3 could suppress the inflammatory response.

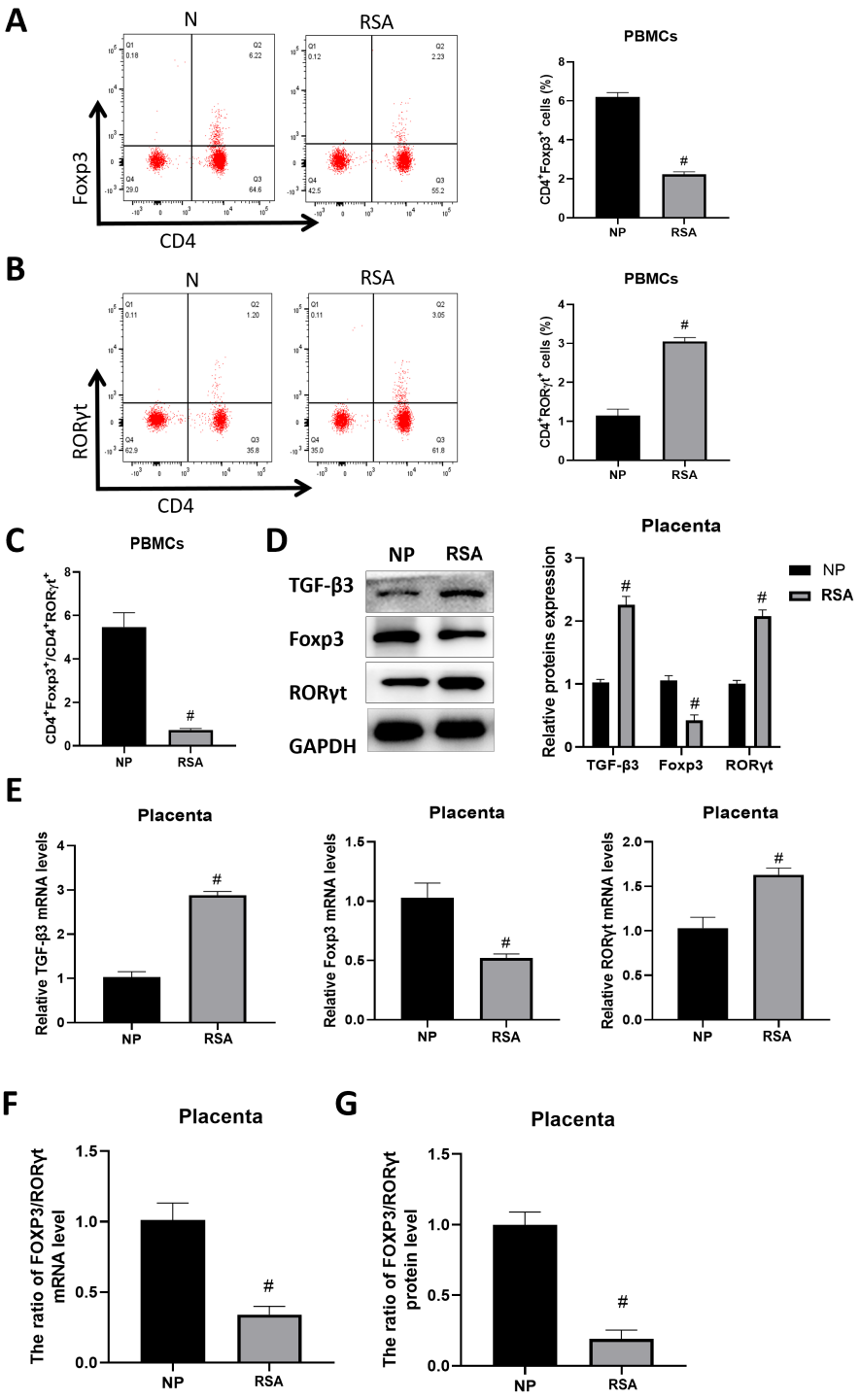

Fig. 1. TGF-β3 level was increased while Treg/Th17 was decreased in RSA. (A) Evaluation and quantification of Tregs using flow cytometry. (B) Evaluation and quantification of Th17 cells using flow cytometry. (C) Computation of the Treg/Th17 ratio in NP and RSA groups. (D) Western blotting to detect protein levels of TGF-β3, Foxp3, and RORγt in the placenta from the NP and RSA groups. (E) qRT-PCR analysis to measure mRNA levels of *TGF-β3*, *Foxp3*, and *RORγt* in the placenta from the NP and RSA groups. (F) Calculation of *Foxp3/RORγt* mRNA ratio in the NP and RSA groups. (G) Calculation of Foxp3/RORγt protein ratio in the NP and RSA groups. N = 10. $^{\#}p < 0.05$ vs NP. Abbreviations: NP, normal pregnancy; RSA, recurrent spontaneous abortion; TGF-β3, transforming growth factor β3; Treg, regulatory T cell; Th17, T helper 17; Foxp3, forkhead box protein P3; RORγt, retinoic-acid-receptor-γt; qRT-PCR, reverse-transcription quantitative polymerase chain reaction; PBMCs, Peripheral Blood Mononuclear Cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

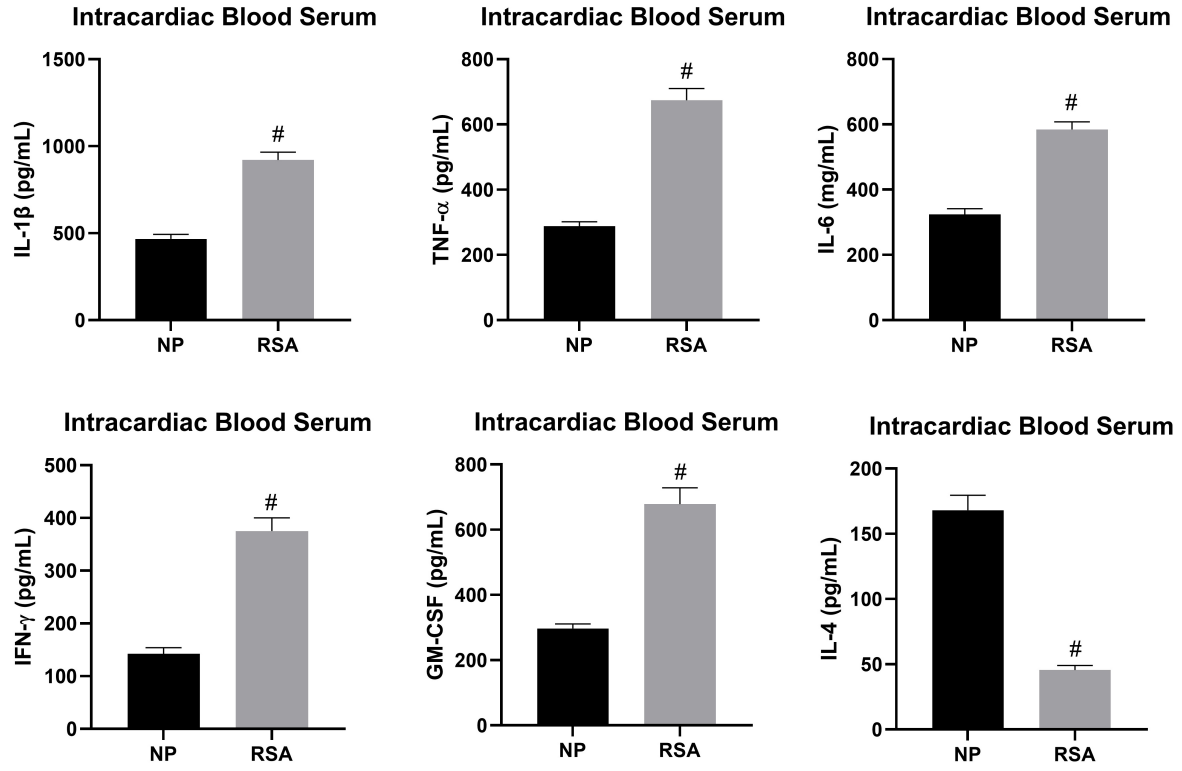

Fig. 2. Heightened inflammatory response in the mouse model of RSA. The levels of IL-1β, TNF-α, IL-6, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 in intracardiac blood serum of the RSA mouse model were measured. N = 10. $^{\#}p < 0.05$ vs NP. Abbreviations: IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

Identification of CD4$^+$ T Cells

First, we identified the isolated CD4$^+$ T cells morphologically, as shown in **Supplementary Fig. 1A**. The morphological identification was accurate. The results in **Supplementary Fig. 1B** show the flow cytometry identification, indicating that CD4$^+$ cells (98.6%) are positively expressed.

Discussion

The delicate immunological tolerance at the maternal-fetal interface, which regulates immune activities across the placenta such as the invasion of maternal tissues by the allogeneic fetal trophoblasts, as well as the immune protection against a range of pathogens, is essential for maintaining a successful pregnancy. Several pregnancy-related problems, such as RSA, preeclampsia, and fetal intrauterine growth restriction, are thought to be linked to the disruption of this immunological balance [35].

TGF-β3 is a growth factor autonomously manufactured and released by the developing pathogenic Th17 cells [22]. The pro-inflammatory properties of TGF-β3 may be mitigated by IL-10, which is produced by LAG3$^+$ Treg since it severely inhibits Th17 growth and function [36]. Here, we intended to investigate how TGF-β3 affects Th17 cells and Tregs during pregnancy. In the *in vitro* experiments, we studied the effect of TGF-β3 on Tregs and Th17 production, as well as Th17 plasticity. We discovered that the TGF-β3 level was elevated in mice with RSA. Additionally, TGF-β3 knockdown was found to further disrupt the Treg/Th17 imbalance, offering fresh insights into TGF-β3 function in RSA.

We also examined the correlation between TGF-β3 expression and the proliferative capability of T cells to further understand their connection. However, the lack of a relationship between TGF-β3 expression and the Tregs percentage most likely suggests that increased TGF-β3 expression inhibits the proliferation of Tregs. Although Tregs proliferation may have a negligible effect on the overall number of Treg cells in the NP and RSA groups, TGF-β3 expression was inversely correlated with Th17 cell percentage in RSA as compared to NP. However, the proportion of TGF-β3$^+$ Th17 cells was positively correlated with the ability of Th17 cells to proliferate in both NP and RSA settings. Th17 cell proliferation in RSA was weakly inhibited by lower TGF-β3 expression, which, if elevated, increased the proportion of Th17 cells.

Immune homeostasis is hinged on the balance between Tregs and Th17 cells [37]. Foxp3 overexpression in Tregs and RORγt upregulation in Th17 cells are essential for the development of Tregs and Th17 cells, respectively [32]. Our findings showed that TGF-β3 could influence the Treg/Th17 imbalance by promoting Th17 cell differentiation, as opposed to Tregs differentiation, during

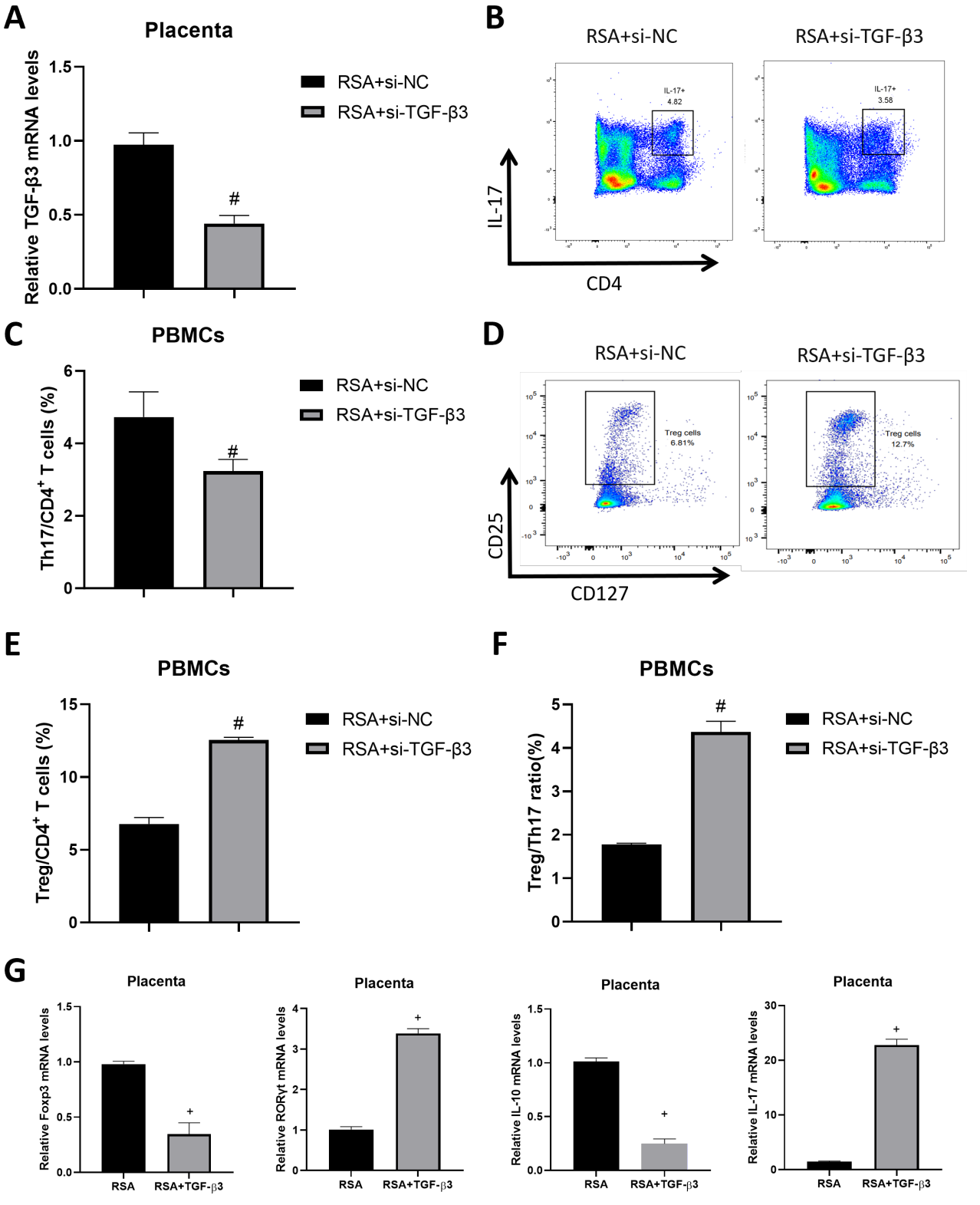

Fig. 3. TGF-β3 silencing restored the normal Treg/Th17 ratio in RSA model mice. (A) qRT-PCR-based assessment of si-NC or si-TGF-β3 transfection. (B–F) Analyses of Th17 ($CD4^+$ $IL\text{-}17^+$) cell percentage (B,C), Treg ($CD4^+$ $CD25^+$ $CD127^-$) cell percentage (D,E), and the ratio of Treg/Th17 (F). (G) Measurement of the mRNA levels of *Foxp3*, *RORγt*, *IL-10*, and *IL-17*. N = 10. $^{\#}p < 0.05$ vs RSA+si-NC; $^{+}p < 0.05$ vs RSA.

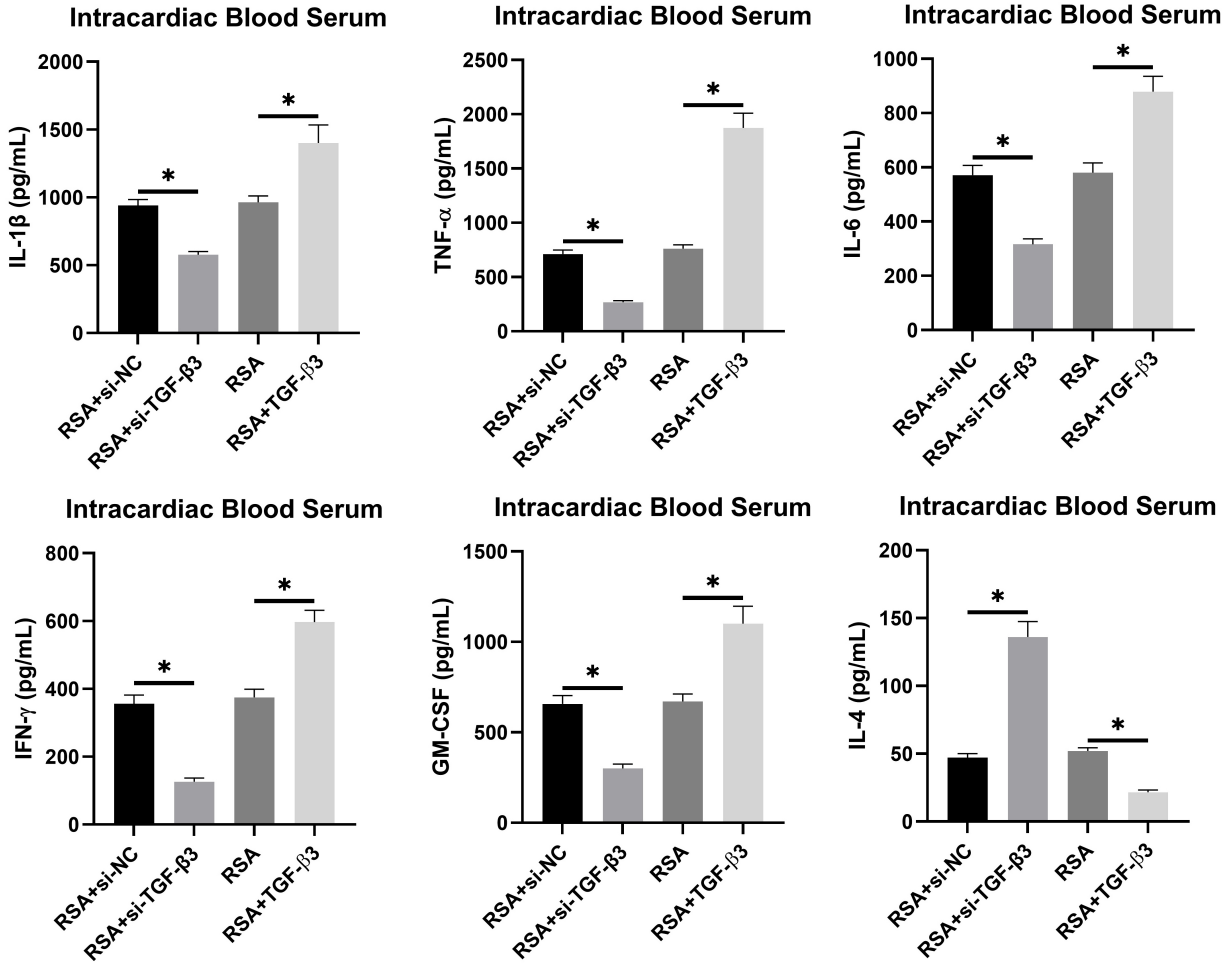

Fig. 4. TGF-β3 silencing suppressed inflammatory response in RSA mice. The levels of IL-1β, TNF-α, IL-6, IFN-γ, GM-CSF, and IL-4 in intracardiac blood serum of RSA mice were measured. N = 10. *$p < 0.05$.

mouse pregnancy. RSA may occur due to TGF-β3 pathway malfunction that upsets the balance between Tregs and Th17 cells. It has been reported that in an inflammatory microenvironment, immunosuppressed Foxp3$^+$ Tregs undergo a steady decrease in Foxp3 expression, eventually transdifferentiating into Th17 cells, which may contribute to the pathophysiology of illnesses [32,38].

The results of this study indicate an upregulation of TGF-β3 in RSA and its association with the imbalance of Tregs and Th17 cells. The imbalance of Treg/Th17 caused by TGF-β3 knockdown sheds light on the potential role of TGF-β3 as a therapeutic target for RSA, providing an important theoretical basis for developing treatment strategies targeting TGF-β3. Of note, TGF-β3 plays a crucial role in the immune system, regulating the function and differentiation of Tregs and Th17 cells. Therefore, by modulating TGF-β3 levels, it is possible to modulate the balance of Tregs and Th17 cells, thereby regulating immune responses and improving the therapeutic outcomes of RSA. Thus, targeting TGF-β3 may provide a new direction for personalized treatment strategies. Accordingly, coupled with measuring the TGF-β3 levels, formulating individualized treatment plans based on the patient's immune status and clinical presentations, it is anticipated to improve treatment efficacy and reduce the rates of adverse effects. Although further clinical research is warranted to validate its safety and efficacy, treatment strategies targeting TGF-β3 have promising clinical application prospects. This approach may become an important component of future RSA treatment, providing patients with more effective treatment options and improving their quality of life.

Furthermore, RSA mice had a greater proportion of IL-17$^+$ Tregs. Rather than the general Tregs proportion, the proportion of IL-17$^+$ Tregs was favorably correlated with the proportion of Th17 cells in two groups. These findings suggested that, particularly in RSA, Tregs may produce IL-17 under inflammatory conditions, thereby contributing to the pathophysiology of the disorder by expanding the pool of Th17 cells.

Our study provides invaluable insights into the role of TGF-β3 in the imbalance of Tregs and Th17 cells in RSA. However, it is important to acknowledge several limitations of this study. Firstly, this study utilized an animal model to unravel the impact of TGF-β3 on the Treg/Th17

balance in RSA. While animal studies could provide insightful findings about the relevant underlying biological mechanisms, we are of the opinion that the differences in immune responses between humans and mice could limit the generalizability of the current set of findings to human populations. Secondly, this study employed a siRNA-mediated knockdown approach to investigate the effects of TGF-β3 on Treg/Th17 balance in RSA mice. Although this approach allowed for the assessment of the direct role of TGF-β3, it does not facilitate a full deciphering of the complex regulatory mechanisms of TGF-β3 in RSA. Additionally, the transient effects of siRNA-mediated knockdown may not fully translate to the long-term effects of TGF-β3 modulation in RSA. Thirdly, our study primarily focused on the Treg/Th17 balance and its modulation by TGF-β3 in RSA but did not explore other potential contributing factors or pathways involved in the pathophysiology of RSA. Thus, further studies are needed to elucidate the comprehensive mechanisms underlying RSA, including the involvement of additional immune cell subsets, cytokines, and signaling pathways. Lastly, we investigated the effects of TGF-β3 modulation on the inflammatory response in RSA by primarily assessing systemic cytokine levels in mouse serum, instead of in human specimens. In view of these shortcomings, further research is warranted to validate our findings—grounded in the potential therapeutic relevance of targeting TGF-β3 in RSA through the restoration of the normal Treg/Th17 balance—in human studies, in which the aforementioned limitations should be sufficiently addressed to expand the generalizability of research findings. Future studies could also benefit from examining tissue-specific immune responses and inflammatory mediators at the maternal-fetal interface to deepen our understanding of the immunological mechanisms involved in RSA.

Conclusions

In summary, our findings showed that aberrant TGF-β3 expression may be linked to Treg/Th17 imbalance in mice with RSA.

Availability of Data and Materials

The corresponding author will provide the data that underpin the study's conclusions with a reasonable application.

Author Contributions

LP designed the study; all authors conducted the study; MLY and XYL collected and analyzed the data. XYL participated in drafting the manuscript, and all authors contributed to the critical revision of the manuscript for important intellectual content. All authors gave final approval of the version to be published. All authors participated fully in the work, took public responsibility for appropriate portions of the content, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or completeness of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

This study has been approved by the Experimental Animal Ethics Committee of the First Hospital of Hunan University of Chinese Medicine (Approval No: ZYFY-20230319-003).

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537193.22.

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