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Original Research

Toll-Like Receptor 4/p65 Signalling Pathway Mediates Recurrent Spontaneous Abortion by Regulating Forkhead Box Protein P3

Caiqun Huang[1,*], Ying Lyu[2], E Fen Cheng[2]

[1]Central Laboratory and Precision Medicine Center, Affiliated Jinhua Hospital, Zhejiang University School of Medicine, 321000 Jinhua, Zhejiang, China
[2]Departments of Obstetrics and Gynecology, Affiliated Jinhua Hospital, Zhejiang University School of Medicine, 321000 Jinhua, Zhejiang, China
*Correspondence: syll_tracy@126.com (Caiqun Huang)

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Abstract

Background: Although multiple factors contribute to recurrent spontaneous abortion (RSA), recent studies have highlighted a role for regulatory T cells (Tregs) in maintaining immune tolerance during pregnancy. Understanding the intricate relationship between RSA and Tregs offers promising avenues for diagnosis and therapeutic strategies in reproductive medicine. **Methods**: We successfully established a mouse model of RSA, and all animals were euthanized on gestational day 13. Uterine horns were examined upon dissection to determine the number of viable fetuses and resorption sites, after which the post-implantation loss rate (abortion rate) was calculated. Placental morphology was evaluated via hematoxylin and eosin staining, whereas gene expression was analysed by immunohistochemistry. Treg abundance was determined by flow cytometry. Gene expression and cytokine levels in EL4 cells and patient samples were evaluated by quantitative real-time polymerase chain reaction (qPCR) and Western blotting. **Results**: The abortion rate of RSA mice was 31.64%. In addition, Treg numbers were reduced in both RSA mice and patients, while the expression of Toll-like receptor 4 (TLR4) and p65 was upregulated, further suppressing forkhead box protein P3 (FOXP3) expression. Blocking p65 expression with small interfering RNA (siRNA) targeting p65 prevented the lipopolysaccharide (LPS)-induced downregulation of FOXP3 in EL4 cells. The TLR4 inhibitor IAXO102 was ineffective at increasing FOXP3 expression in EL4 cells following p65 overexpression p65. **Conclusions**: The results of this study suggest that activation of the TLR4/p65 signalling in RSA inhibits FOXP3 expression and contributes to abortion. Importantly, the findings indicate that Treg suppression in RSA is p65-dependent.

Keywords: recurrent spontaneous abortion; regulatory T cells; forkhead box P3; toll-like receptor 4; nuclear factor kappa-B

1. Introduction

Recurrent spontaneous abortion (RSA), or recurrent miscarriage, is defined as three or more consecutive pregnancy losses prior to 28 weeks' gestation [1]. This condition presents substantial physical and emotional challenges, necessitating a multidisciplinary approach to care [2]. Advances in our understanding of the mechanisms involved, particularly those relating to maternal immune dysregulation and cytokine imbalances, may reveal novel therapeutic targets and improve outcomes [3]. Continued research is essential to develop effective interventions and enhance reproductive health.

Regulatory T cells (Tregs) are essential for maintaining peripheral tolerance, preventing autoimmunity, and establishing fetal tolerance during pregnancy [4,5]. A reduction in Treg number and function mediates fetal rejection, a phenomenon that is reversible in animal models via Treg transplantation [6]. Forkhead box protein P3 (FOXP3), a key transcriptional regulator of Treg development, has also been associated with reproductive failure when underexpressed in endometrial tissue [7]. This study found that peripheral blood Treg levels were significantly decreased in patients with RSA.

Toll-like receptor 4 (TLR4) recognises pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), initiating signalling that causes nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) subunit p65 and subsequent inflammatory gene transcription [8–12]. The TLR4/NF-κB pathway modulates Tregs in a context-dependent manner; it can either suppress Treg function and promote effector T-cell responses [13–16] or support Treg differentiation to resolve inflammation [17,18]. This complex interplay suggests that the expression of FOXP3, which is central to Treg identity and function, may be regulated by the TLR4/p65 signalling pathway.

To explore the role of immune dysregulation in RSA, we used a mouse model and human specimens. We discovered that the TLR4/p65 pathway is hyperactive in RSA and directly inhibits FOXP3, which is a key regulator of protective Tregs. By using a pathway agonist (LPS) and antagonist (IAXO102), as well as genetically knocking down and overexpressing p65, we demonstrated, at a mechanistic level, that targeting this pathway modulates FOXP3 expression. These findings reveal a novel therapeutic strategy for preventing miscarriage.

2. Materials and Methods

2.1 Patient Recruitment and Inclusion/Exclusion Criteria

This study recruited 20 patients with RSA and 20 healthy control participants. Inclusion criteria for the RSA group: Females aged 20–45 years who meet the criteria for an RSA diagnosis, with a current intrauterine pregnancy confirmed via ultrasound. Both partners must provide informed consent and sign the consent form. Exclusion criteria included known causes of miscarriage, anatomical abnormalities of the reproductive tract, uncontrolled endocrine or metabolic disorders, autoimmune diseases, prothrombotic states, recent acute infections and other major systemic diseases. The control group comprised healthy women in early pregnancy who had voluntarily terminated their pregnancy for non-medical reasons (the normal early pregnancy abortion group). The gestational age of this group was matched with that of the RSA group, and all the RSA group's exclusion criteria were applied. All participants provided informed consent and signed informed consent forms.

2.2 Sample Collection and Processing

This study involved collecting products of conception tissue and peripheral blood.

On the day of the ultrasound-guided abortion or surgical procedure, the tissue was obtained using sterile instruments and immediately divided into two portions. One portion was placed in 4% paraformaldehyde for fixation, followed by paraffin embedding, histological sectioning and immunohistochemical analysis. The other portion was placed in a sterile, enzyme-free cryopreservation tube, rapidly frozen in liquid nitrogen and transferred to –80 °C storage for subsequent molecular biology experiments, such as protein/RNA extraction, Western blot and quantitative real-time polymerase chain reaction (qPCR). Peripheral blood: Collect 5–10 mL of fasting venous blood from patients in the morning using EDTA anticoagulant tubes (Bacet, Wenzhou, Zhejiang, China). Centrifuge at 3000 rpm for 15 minutes at 4 °C. Aspirate the plasma from the supernatant, divide it into smaller portions, and store it at –80 °C. All sample tubes were labelled with unique, traceable codes (concealing patient names) and a sample information database was created. This database recorded the following information for each sample: ID, patient group, age, parity, number of miscarriages, gestational age, collection date, processing method and storage location. Biological samples were stored in –80 °C freezers or liquid nitrogen tanks, and storage equipment operation was regularly checked.

2.3 Mice and Mating

A total of forty-five 6-week-old mice, comprising thirty CBA/J, five DBA/2, ten BALB/c, were purchased from GemPharmatech™ (Nanjing, Jiangsu, China) and housed in stainless steel cages with ad libitum access to food and water. They were kept under controlled conditions of temperature (22 $\pm$ 2 °C) and humidity (50 $\pm$ 5%) with a light/dark cycle of 12/12 h. To generate a normal pregnancy model, female CBA/J mice were mated with male BALB/c mice at a 2:1 ratio. For the RSA model, female CBA/J mice were paired with male DBA/2 mice at a 2:1 ratio. The next morning, the presence of a vaginal plug was used to mark the start of gestational day 0 (G0). On G5, normal pregnant mice received an injection of either RU486 (5 mg/kg; Aladdin, Shanghai, China) or an equivalent volume of phosphate-buffered saline (PBS). All mice were humanely euthanised on G13 under sustained isoflurane anaesthesia (3.0–4.0%) by trained personnel using cervical dislocation. Pregnancy outcomes were assessed by examining the uterine horns. Necrosis and haemorrhage of the foetuses and placentas were evaluated by macroscopic inspection. The miscarriage rate was determined by dividing the number of resorption sites by the total number of implantation sites.

2.4 Culture of Mouse Thymic Lymphoma Cells (EL4)

The EL4 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (TCM41, Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was refreshed every three days, and the cells were subcultured when they reached 80% confluence. Mycoplasma testing was negative. The EL4 cells have been authenticated by STR profiling.

2.5 Haematoxylin & Eosin Staining and Immunohistochemistry

The uterine tissue segments were fixed in 4% paraformaldehyde, dehydrated using a graded ethanol series and embedded in paraffin wax Thin sections were stained with haematoxylin and eosin (H&E) and examined using the Zeiss light microscope (Oberkochen, Germany). For immunohistochemistry, the placental sections were deparaffinised and treated with 3% hydrogen peroxide to block endogenous peroxidase. They were then incubated with goat serum to minimise non-specific binding. An overnight incubation of the slides was performed at 4 °C with anti-TLR4 antibody (1:500, 19811-1-AP; proteintech, Wuhan, Hubei, China), anti-p65 antibody (1:200, 3033; Cell Signaling Technology, Danvers, MA, USA), and anti-FOXP3 antibody (1:500, 22228-1-AP; proteintech, Wuhan, Hubei, China). After three washes in 0.01 M PBS, the samples were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000, TA373083L; OriGene, Beijing, China) for 30 min at 37 °C. Immunostaining was visualised using 3,3′-diaminobenzidine (DAB) and the sections were counterstained with Mayer's haematoxylin (ZSBG-BIO, Beijing, China). The sections were then dehy-

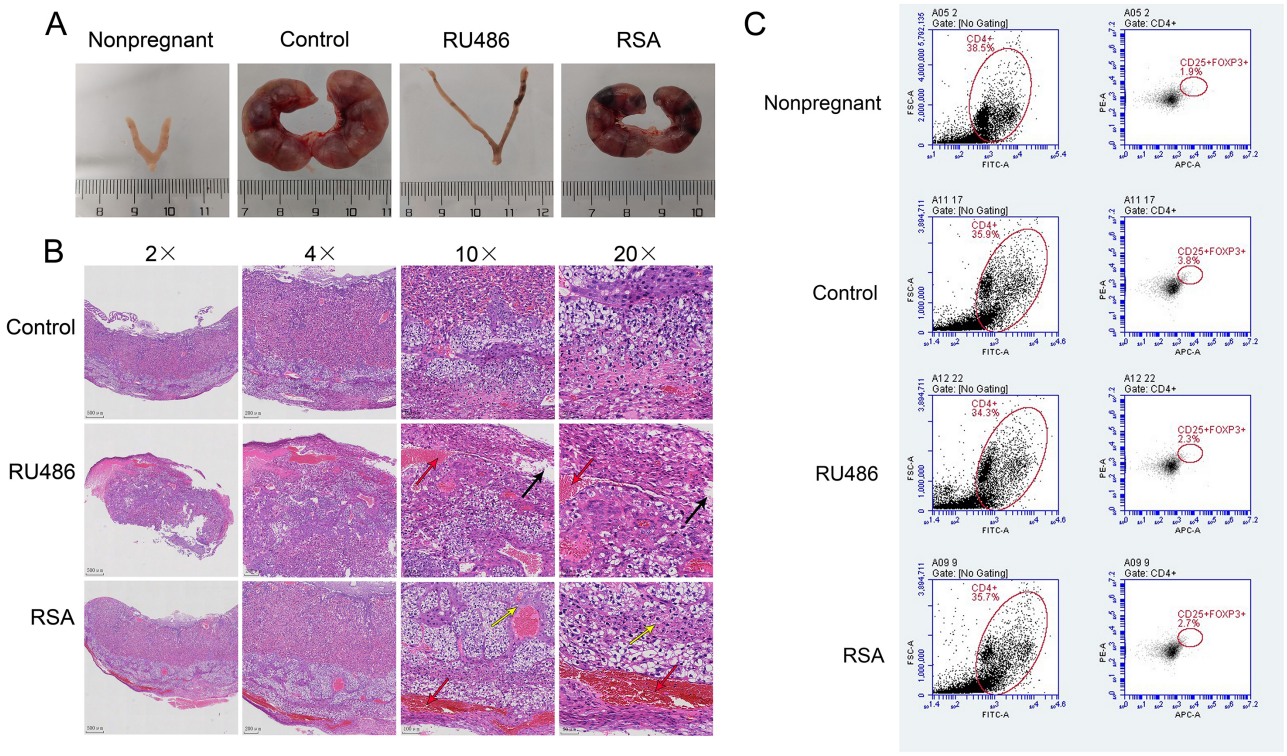

Fig. 1. Reduced Treg cell expression in RSA mice. (A) Representative uterine specimens were collected from both normal and RSA-affected pregnancies at G13. (B) Histopathological evaluation using H&E staining was performed on maternal–fetal interface sections from the corresponding experimental groups. The placental structure was clearly defined in the RSA group, but disorganised in the RU486 group. Black arrows mark loose oedema, yellow arrows indicate necrotic cells, red arrows indicate bleeding. 2× Scale bar: 500 μm; 4× Scale bar: 200 μm; 10× Scale bar: 100 μm; 20× Scale bar: 50 μm. (C) Flow cytometry to detect the proportion of CD4+CD25+FOXP3+ T cells. H&E, haematoxylin and eosin.

drated, cleared and mounted. Immunohistochemical staining for TLR4, p65 and FOXP3 was quantified using a standardised H-scoring system [19].

2.6 Detection of Treg Frequency

The frequency of Tregs was determined using flow cytometry to analyse single-cell suspensions from mouse spleens and human peripheral blood. Spleens were harvested from each group and processed into single-cell suspensions using a previously established protocol [20]. Human peripheral blood mononuclear cells were isolated using Ficoll density gradient centrifugation. To quantify the CD4+CD25+FOXP3+ T cell population, mouse and human Tregs staining kits (88-8111-40 and 88-8999-40, respectively, eBioscience, San Diego, CA, USA) were employed in accordance with the manufacturers' instructions. Data are presented as the percentage of Tregs within the total cluster of differentiation 4 (CD4)+ T cell population [21].

2.7 Western Blot Analysis

Total protein was extracted from the abortion products of patients with RSA, and the protein concentration was determined by the bicinchoninic acid (BCA) method. Immunoblotting was performed using standard methods. TLR4 antibody (1:500, 19811-1-AP; proteintech, Wuhan, Hubei, China), TLR4 (1:2000, ab13556; Abcam, Cambridge, UK), p65 (1:1000, 3033; Cell Signaling Technology, Danvers, MA, USA), and FOXP3 (1:2000, ab215206; Abcam, Cambridge, UK) were used to detect the proteins. GAPDH (1:50,000, 60004-1-Ig, proteintech, Wuhan, Hubei, China) was used as an internal control to normalise protein loading. Anti-rabbit (1:5000) secondary antibodies were conjugated with HRP (P6782; Sigma-Aldrich, Shanghai, China). HRP-labeled Goat Anti-Mouse IgG (H + L) (1:2000, A0216, Beyotime, Shanghai, China) were used. Following separation, the membranes were trimmed according to the migration of a colorimetric protein marker. Immunoreactive bands were detected using an enhanced chemiluminescence system (Merck, Darmstadt, Germany) and quantified using densitometry with ImageJ software (Version 1.54; National Institutes of Health, Bethesda, MD, USA).

2.8 Quantitative PCR

Total RNA was extracted from the abortion products of patients with RSA using Trizol reagent (15596026; In-

vitrogen, Carlsbad, CA, USA) for qPCR analysis. The expression of three target genes (TLR4, p65, FOXP3) and the housekeeping gene (β-*actin*) was assessed using 20 µL reactions containing 10 µL of Real-Time PCR SYBR Green Master Mix (RR036A; Takara, Kyoto, Japan), 0.3 µL of each primer and 7.4 µL of RNase-free water. Amplification was performed on a Cobas z480 System (Roche, Rochester, NY, USA) under the following conditions: Initially, the samples were heated for 30 seconds at 94 °C, followed by 40 cycles of 5 seconds at 94 °C and 10 seconds at 60 °C, then 5 seconds at 95 °C, 1 minute at 60 °C and 30 seconds at 50 °C. To assess the mRNA transcript levels, the expression of target genes was normalised to the housekeeping gene β-*actin*. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The Ct values of the target genes were normalized to the β-*actin* ($\Delta\Delta$Ct). The $\Delta\Delta$Ct value was then determined by comparing the ΔCt of each sample to the mean ΔCt of the calibrator group. All qPCR reactions were conducted in triplicate. The sequences for all primers used in this study are listed in **Supplementary Table 1**.

2.9 RNA Interference and Overexpression

Small interfering RNAs (siRNAs) were synthesised by Genepharma (Shanghai, China). A universal control siRNA was used as a non-specific control. EL4 cells were transfected with the siRNA duplex using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The p65 genes were amplified and cloned into a pcDNA3.1 vector (Novagen, Madison, WI, USA). Transfection of EL4 cells was achieved using the Amaxa Nucleofector system (Thermo Fisher Scientific, Waltham, MA, USA).

2.10 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA). Student's *t*-test was used for intergroup comparisons, and one-way ANOVA was used for multiple comparisons. Tukey's post-hoc test was then used to identify specific differences between groups. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1 Decreased CD4+CD25+FOXP3+ T-Cell Numbers in an RSA Mouse Model

To evaluate the divergence in gestational outcomes in RSA mice, both normal control and RSA pregnancy models were established, as previously described [22]. In the normal pregnancy group, mice received intraperitoneal injections of either PBS or RU486 during early gestation (G5). All mice were euthanised at G13. In the normal pregnancy model, administration of RU486 induced an abortion rate of 97.53%, which was markedly higher than the 6.25% observed in the PBS-treated control group, confirming the efficacy of RU486 in establishing an early pregnancy loss model. However, the miscarriage rate in the RSA pregnant mouse model was 31.64% (Table 1). The RSA group experienced fetal and placental necrosis and haemorrhage, whereas control mice had almost no obvious fetal abnormalities (Fig. 1A). H&E staining was performed on uterine sections from the control, RU486-treated, and RSA model groups (Fig. 1B). The maternal–fetal interface in the RSA mouse model displayed pronounced haemorrhagic changes and inflammatory characteristics. This result confirmed that the RSA model was successfully constructed.

Given the established role of CD4+CD25+FOXP3+ T cells in maternal–fetal immune tolerance, their prevalence among splenocytes was quantified using flow cytometry. The proportion of these Tregs was significantly reduced in both the RSA and RU486 groups. However, it was still higher than that of the non-pregnant group (Fig. 1C). The statistical graph for Fig. 1C is shown in **Supplementary Fig. 2**. These findings suggest that impaired expansion or maintenance of CD4+CD25+FOXP3+ T cells is associated with pregnancy loss in the RSA model.

3.2 Reduced Treg Expression Induced by an RSA Mouse Model Is Related to the TLR4/NF-κB Signalling Pathway

FOXP3, a pivotal transcription factor governing the development and function of Tregs, plays a critical role in diverse immunological processes including immune homeostasis, autoimmune pathogenesis, infectious responses, and tumour immune evasion [23]. The expression of TLR4, p65, and FOXP3 in placenta tissue was assessed using Western blotting and immunohistochemistry. As shown in Fig. 2 ($p < 0.05$), the RSA model exhibited increased expression levels of TLR4 and p65, while FOXP3 expression decreased. In the RU486 model, however, only a decrease in FOXP3 expression was observed. These data indicate that a reduction in Tregs is associated with TLR4/p65 activation in the RSA mouse model.

3.3 Patients With RSA Have Reduced Tregs and High TLR4 and p65 Expression

Activation of the TLR4 signalling pathway significantly contributes to the downregulation of FOXP3 expression [24]. To verify the link between RSA and Treg reduction, Treg levels in the peripheral blood of patients with RSA were assessed using flow cytometry. Compared with the control group, the number of Tregs in patients with RSA was reduced (Fig. 3A and **Supplementary Fig. 3**, $p < 0.001$). We used RT-PCR to further validate that TLR4 and p65 were highly expressed, whereas there was low expression of FOXP3 in the abortion products of patients with RSA (Fig. 3B, * for $p < 0.05$, ** for $p < 0.01$). Western blot analysis of TLR4, p65, and FOXP3 protein expression yielded the same results (Fig. 3C, $p < 0.05$). These findings suggest that Treg reduction in patients with RSA is associated with TLR4/p65 activation.

Table 1. Comparisons of fetal resorption rates in pregnant mice.

Group	Number of mice	Surviving fetuses	Resorbed fetuses	Resorption rate (%)
Control	10	75	5	6.25
RU486	10	2	79	97.53***
RSA	10	54	25	31.64*

The abortion rate was determined by dividing the number of abortion sites by the total number of implantation sites. Group comparisons for abortion rates were performed using Student's *t*-test. Statistical differences between groups are shown as follows: Compared with the control group, *$p < 0.05$, ***$p < 0.001$. The statistical graph for Table 1 is shown in **Supplementary Fig. 1**. RSA, recurrent spontaneous abortion.

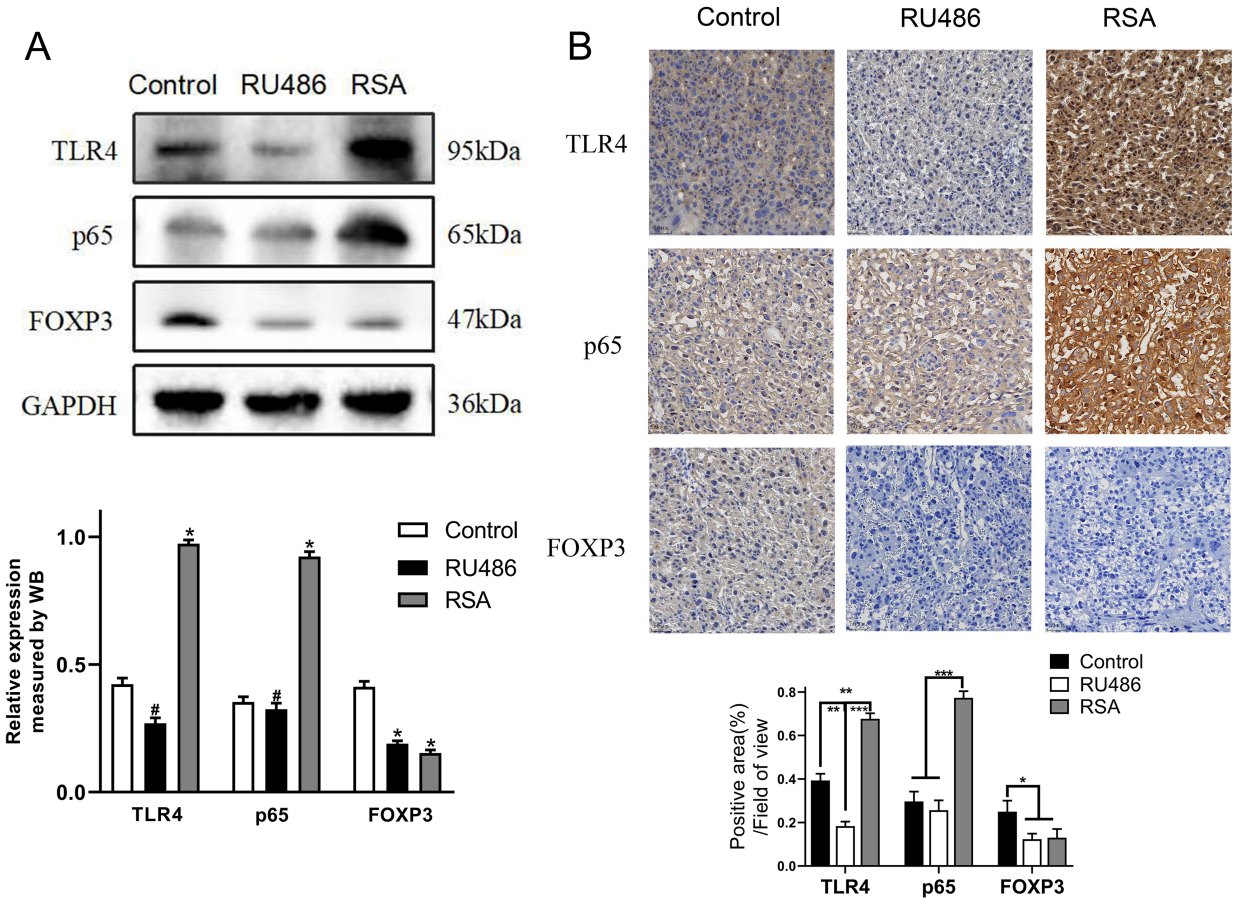

Fig. 2. Regulation of the TLR4/p65/FOXP3 signalling pathway in RSA mice. (A) The protein expression levels of TLR4, p65, and FOXP3 in placental tissue were assessed by Western blotting. (B) TLR4, p65, and FOXP3 was detected by immunohistochemistry. Scale bar: 50 µm. *: Compared with the control group, $p < 0.05$; **: Compared with the control group, $p < 0.01$; ***: Compared with the control group, $p < 0.001$; #: Compared with the control group, $p > 0.05$. TLR4, Toll-like receptor 4; FOXP3, forkhead box protein P3.

3.4 RSA-Induced Downregulation of FOXP3 in EL4 Cells Is Dependent on p65

To investigate the underlying molecular mechanisms, we employed the EL4 cell line, a model characterised by strong inducible FOXP3 expression upon stimulation, while retaining numerous intrinsic T-cell properties [25]. To assess the involvement of p65 in RSA-induced FOXP3 downregulation, we initially stimulated the TLR4/p65 pathway using the agonist LPS, and subsequently inhibited p65 expression via siRNA targeting p65 (si-p65), with a non-targeting control siRNA (si-NC) serving as the negative control. Moreover, the TLR4 inhibitor IAXO102 was chosen to inhibit the TLR4/p65 signalling pathway, followed by overexpression of p65 with pcDNA3.1-p65, the corresponding empty vector control (pcDNA-NC) was used as the negative control. Compared to the si-NC group, transfection with si-p65 significantly reduced p65 protein expression (Fig. 4A, $p < 0.05$). Conversely, transfection

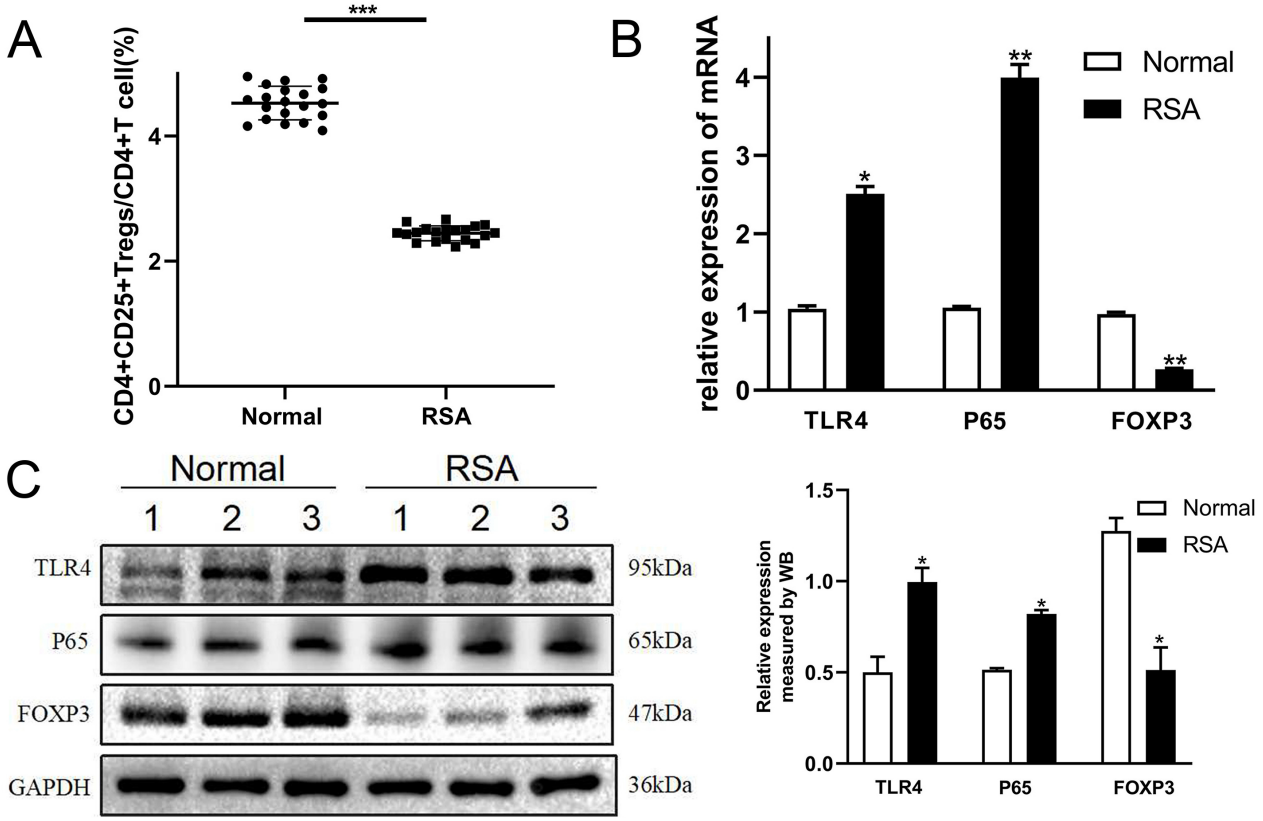

Fig. 3. Expression levels of TLR4, p65, and FOXP3 in patients with RSA. (A) Percentage of CD4+CD25+FOXP3+ T cells in peripheral blood was assessed using flow cytometry. (B) qPCR was performed to measure TLR4, p65, and FOXP3 transcript levels in decidual tissue. (C) Protein expression of TLR4, p65, and FOXP3 were detected by Western blotting. *: compared with the normal decidua, $p < 0.05$; **: Compared with the normal decidua, $p < 0.01$; ***: Compared with the normal peripheral blood, $p < 0.001$.

with the pcDNA3.1-p65 plasmid substantially increased p65 protein expression compared to the pcDNA-NC group (Fig. 4B, $p < 0.05$). These results verified the efficiency of p65 knockdown and overexpression. The results also demonstrated that LPS stimulation of EL4 cells significantly increased TLR4 and p65 expression while downregulating FOXP3. Furthermore, knockdown of p65 prevented LPS-induced suppression of FOXP3 expression (Fig. 4A, $p < 0.05$). Pharmacological inhibition of TLR4 using IAXO102 inhibited TLR4 and p65 expression and significantly upregulated FOXP3. However, this enhanced FOXP3 expression induced by IAXO102 was inhibited by pcDNA3.1-p65 (Fig. 4B, $p < 0.05$). Taken together, these results indicate that p65 is critical for the TLR4-mediated regulation of FOXP3 in EL4 cells.

4. Discussion

RSA is a multifactorial disorder that involves genetic, anatomical, hormonal, immunological and environmental factors [26]. Further research into its aetiology is essential for improving diagnosis, risk assessment, and clinical management [27]. Furthermore, the substantial medical and psychological burden imposed by RSA highlights the need for further investigation to alleviate its impact on patients and healthcare systems [28].

In the case of RSA, disturbances of the immune tolerance mechanism may lead to miscarriage [29]. Recent research identified Tregs as a subpopulation with immunosuppressive functions [30]. FOXP3 serves as a lineage-defining transcription factor that governs the development and suppressive function of Tregs. Treg-mediated immunoregulation is critically involved in maintaining self-tolerance and preventing autoimmune pathologies, as well as establishing maternal–infant immune tolerance during pregnancy [31]. During normal pregnancy, the frequency of Tregs in peripheral blood rises during early pregnancy. These cells subsequently migrate to the maternal–infant interface, where they respond to various chemokines produced by human chorionic gonadotropin and trophoblast cells [32]. Consistent with previous findings in both animal models and humans, our results reconfirm that Treg depletion is associated with maternal immune dysregulation in conditions such as RSA [33]. We observed a reduction in Tregs in the RSA mouse model, aligning with earlier reports of suppressed Treg levels in the peripheral blood of patients with RSA.

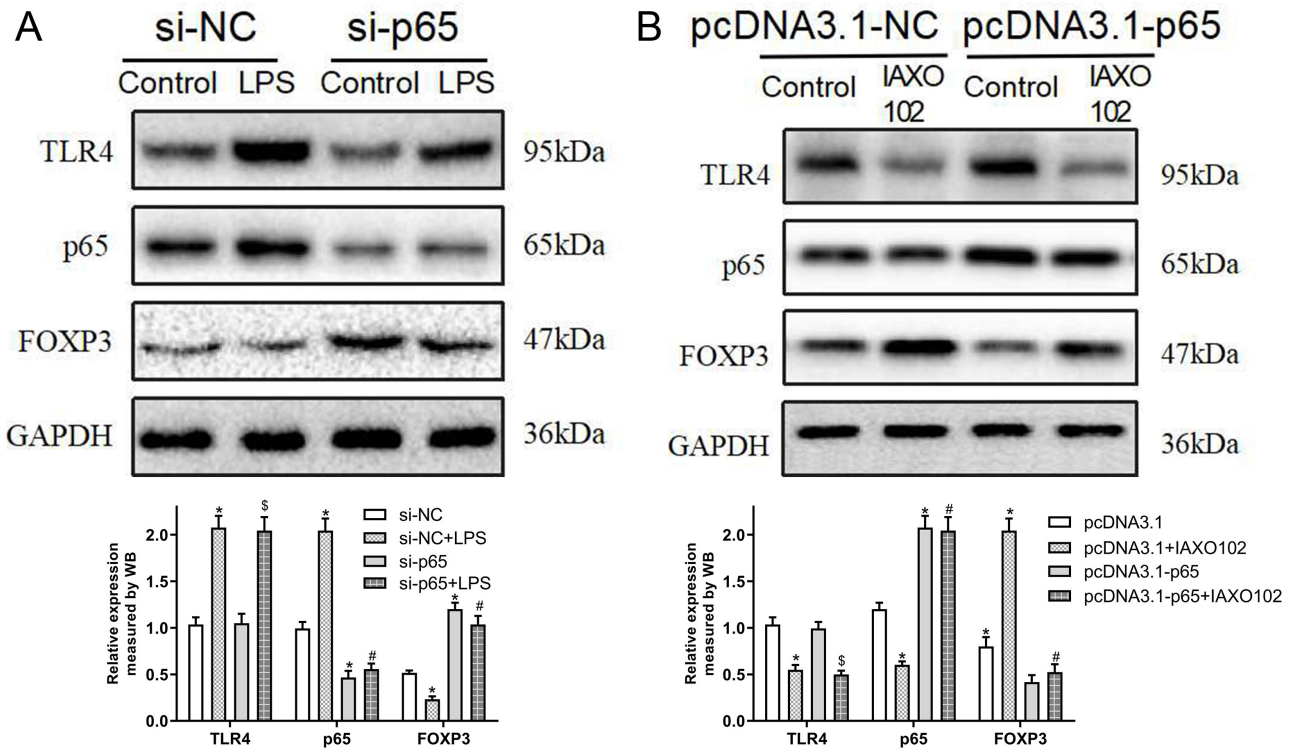

Fig. 4. Downregulation of FOXP3 by TLR4 signalling is p65-dependent. (A) EL4 cells were transfected with p65-target siRNA or control siRNA with or without LPS (TLR4 agonist). Western blotting showed TLR4, p65, and FOXP3 protein expression. *$p <$ 0.05 compared to the si-NC group. \$$p < 0.05$ compared to the si-p65 group. #$p > 0.05$ compared to the si-p65 group. (B) Cells were transfected with pcDNA3.1-p65 or empty vector with or without IAXO102 (TLR4 inhibitor). *$p < 0.05$ compared to the pcDNA3.1-NC group. \$$p < 0.05$ compared to the pcDNA3.1-p65 group. #$p > 0.05$ compared to the pcDNA3.1-p65 group.

As a specific TLR4 agonist, LPS activates TLR4 and its downstream NF-κB signalling pathways, thereby promoting inflammatory responses and cytokine production [34]. TLR4 is expressed in immune cells, trophoblast and decidual cells at the maternal–fetal interface [35]. Disruption of immune tolerance at this interface due to infectious or inflammatory stimuli is often implicated in pregnancy complications [36]. Nevertheless, the specific impact of TLR4 activation on Tregs within the maternal–fetal interface remains to be fully elucidated. Our *in vitro* experiments showed that LPS stimulation upregulated TLR4 and p65 expression and reduced FOXP3 levels, whereas the TLR4 inhibitor IAXO102 inhibited TLR4 and p65 and upregulated FOXP3. These results suggest that, in the context of inflammation-driven pregnancy failure, activation ultimately results in the significant suppression of Treg function via NF-κB-mediated pathways. Preclinical studies have demonstrated the potential of IAXO102, a TLR4 signalling pathway antagonist, to treat multiple diseases by inhibiting key inflammatory pathways such as NF-κB. Further validation of IAXO102's regulatory effects on the TLR4/p65-FOXP3 axis is required in primary Treg cells or RSA animal models to determine its clinical therapeutic potential.

To further investigate the regulation of FOXP3 by p65, we used p65-specific siRNA and overexpression plasmids. We found that p65 knockdown prevented LPS-induced downregulation of FOXP3, and p65 overexpression inhibited IAXO102-induced FOXP3 upregulation. These results suggest that p65 directly regulates FOXP3 expression in this model, providing mechanistic support for the notion that TLR4/NF-κB activation can impair Treg stability and function. Further *in vivo* and *in vitro* studies involving Treg-specific p65 knockout are required to definitively establish a causal relationship between p65 and FOXP3 regulation in Treg cells.

A key limitation of this study is the use of the EL4 cell line, which may not fully replicate the complexity of the maternal-fetal immune microenvironment *in vivo*. Furthermore, extrapolating these findings to human clinical contexts requires validation through *in vivo* models and human tissue studies. Future work should also explore the interactions between trophoblasts and immune cells within pathological microenvironments to better understand the potential for translating TLR4/NF-κB modulation into clinical practice.

5. Conclusions

In summary, this study reconfirms the association between Treg deficiency and recurrent miscarriage, suggesting that the TLR4/NF-κB signalling pathway may be involved in Treg suppression in a p65-dependent manner. While these findings advance our knowledge of the mechanisms of immune dysregulation in recurrent miscarriage, further research is required to establish their clinical significance. Future studies should validate these mechanisms in models that more closely resemble physiological conditions and explore potential therapeutic strategies that target the TLR4 signalling pathway to improve gestational outcomes.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

CH designed the research study and performed the research. EFC provides assistance and guidance for clinical specimen collection, making substantial contributions to data acquisition and analysis. YL analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was reviewed and approved by the Medical Ethics Committee of Jinhua Municipal Central Hospital (Approval No. 2023-149; Jinhua, Zhejiang Province, China). The informed consent was obtained. The study was conducted in accordance with the principles of the Declaration of Helsinki. All animal experiments were conducted in accordance with institutional guidelines and the principles of the 3Rs (Replacement, Reduction, and Refinement). All animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Jinhua Municipal Central Hospital (Approval No. AL-JHYY202327), and all animal experiments were performed following approved animal care and use protocols.

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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/10.31083/CEOG45730.

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