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$CD4^+CD25^+Foxp3^+$ regulatory T cells regulate immune balance in unexplained recurrent spontaneous abortion via the Toll-like receptor 4/nuclear factor-κB pathway

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Abstract

Objective: The present study aimed to evaluate the effects of cluster of differentiation (CD) 4^+CD25^+ forkhead box p3 (Foxp3)$^+$ regulatory T cells (Tregs) on unexplained recurrent spontaneous abortion (URSA) and the associated mechanisms.

Methods: The proportion of $CD4^+CD25^+Foxp3^+$ Tregs and inflammatory cytokine concentrations in the peripheral blood of women with URSA were measured by flow cytometry and enzyme-linked immunosorbent assay, respectively. CBA/JxDBA/2J mating was used to establish an abortion-prone mouse model and the model mice were treated with the Toll-like receptor 4 (TLR4) antagonist E5564 and the TLR4 agonist lipopolysaccharide.

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Results: The proportion of $CD4^+CD25^+Foxp3^+$ Tregs was decreased and the inflammatory response was increased in women with URSA. In the abortion-prone mouse model, E5564 significantly increased the proportion of $CD4^+CD25^+Foxp3^+$ Tregs, decreased the inflammatory response, and increased Foxp3 mRNA and protein expression. Lipopolysaccharide had adverse effects on the abortion-prone model.
Conclusions: These data suggest that $CD4^+CD25^+Foxp3^+$ Tregs regulate immune homeostasis in URSA via the TLR4/nuclear factor-κB pathway, and that the TLR4 antagonist E5564 may be a novel and potential drug for treating URSA.

Keywords
Unexplained recurrent spontaneous abortion, $CD4^+CD25^+Foxp3^+$, regulatory T cells, Toll-like receptor 4, lipopolysaccharide, immune balance

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Introduction

An estimated 1% to 3% of women experience three or more consecutive abortions before 20 weeks of gestation and this is defined as recurrent spontaneous abortion (RSA).[1] Although chromosomal, endocrinological, anatomical, infectious, and autoimmunological abnormalities have been implicated in RSA, its etiology remains unknown. More than 50% of occurrence of RSA does not have a specifically determined cause of abortion[2] and this is called unexplained RSA (URSA). URSA is largely associated with failure of fetomaternal immunological tolerance.[3]

Regulatory T cells (Tregs) play a major role in fetomaternal immunological tolerance. Tregs can suppress an aggressive allogeneic response directed against the fetus and the absence of Tregs leads to failure of gestation due to immunological rejection of the fetus.[4] Cluster of differentiation (CD) 4^+CD25^+ Tregs, a particular subset of T cells, play an important role in development and maintenance of tolerance in peripheral tissues. URSA might be related to abnormal proportions of $CD4^+CD25^+$ Tregs.[5–7] Forkhead box p3 (Foxp3) is an essential transcription factor for induction and development of $CD4^+CD25^+$ Tregs and a unique marker of regulatory T cells.[8] A previous study showed that $CD4^+CD25^+$ Tregs may play an important role in maintaining normal pregnancy and a reduction in $CD4^+CD25^+$ Tregs with lower Foxp3 expression may be involved in the pathogenesis of URSA.[9] Clinical studies have shown that $CD4^+CD25^+Foxp3^+$ Treg expression is significantly decreased in women with URSA compared with normal pregnant women.[10,11] Additionally, the reduced percentage of peripheral $CD4^+CD25^+Foxp3^+$ Tregs during the late follicular phase is associated with failure of artificial insemination by donor sperm.

Toll-like receptor 4 (TLR4) is expressed on Tregs and it is capable of identifying bacterial lipopolysaccharide (LPS). This indicates that LPS may affect Treg function by stimulation of TLR4.[12] LPS binding to TLR4 triggers myeloid differentiation through the primary response gene-88 (MyD88)-independent pathway, leading to subsequent activation of nuclear factor (NF)-κB.[13] NF-κB can effectively induce

the expression of various inflammatory cytokines and stimulate their release from cells.[14] Tregs, characterized by positive expression of CD4, CD25, and Foxp3 ($CD4^+CD25^+Foxp3^+$), contribute to maintenance of immune homeostasis, prevention of autoimmunity, and moderation of the inflammatory response.[15] Previous studies have suggested that TLR4 release leads to excess T-helper type 1 (Th1) cytokines, resulting in imbalance of Th1/Th2 and URSA.[16,17] Therefore, inhibiting the TLR4/NF-κB signaling pathway in $CD4^+CD25^+$ Tregs may have potential therapeutic advantages for inflammation-related diseases, including URSA.

Eritoran tetrasodium (E5564) is a TLR4 antagonist that has been used in clinical trials and can block LPS-mediated activation of NF-κB in TLR4/MD-2-transfected cells.[18] The present study aimed to investigate the effects of $CD4^+CD25^+Foxp3^+$ Tregs on URSA and the associated mechanisms by using E5564 to block and LPS to activate the TLR4/NF-κB pathway.

Methods

Subjects

All of the participants who were prospectively enrolled in the study were outpatients at the Department of Gynecology, University of Chinese Academy of Sciences Shenzhen Hospital (Guangming, Shenzhen, China). All participants were healthy, except for their history of recurrent abortions, and were negative for blocking antibodies. Heparinized elbow venous peripheral blood was obtained from all of the participants at a mean gestational age of 8.2 ± 1.3 weeks. Eighteen uterine villi were collected from the URSA (8 uterine villi) and control (10 uterine villi) groups for routine hematoxylin and eosin (HE) staining.

Animal modeling and intervention

Fifty 8 to 10-week-old female CBA/J ($h\text{-}2^k$) mice, 4 male BALB/c ($h\text{-}2^d$) mice, and 12 male DBA/2J ($H\text{-}2^d$) mice weighing 20 to 25 g were obtained from the Laboratory Animal Center of Jinan University (Guangzhou, China). CBA/JxDBA/2J mating with a high fetal resorption rate was used as the abortion-prone model, whereas CBA/JxBALB/c mating was used as a normal pregnancy model with a low resorption rate. The day at which a copulatory plug appeared was arbitrarily designated as Day 0 of gestation. Four days later, the mice received placebo (vehicle only: 0.9% saline, sham group), E5564 (Eritoran; Career Henan Chemical Co., Zhengzhou, China) (200 μg/mouse intravenously, E5564 group), or *Escherichia coli* LPS (L2880; Sigma-Aldrich; Merck KGaA, Shanghai, China) (3.0 μg/g mouse, LPS group) once every 2 days for 10 successive days (Days 4–14).[18] On day 14 of gestation, 50 pregnant CBA/J mice were anesthetized using intraperitoneal injection of 1% sodium pentobarbital. Peripheral blood was immediately isolated and then the mice were sacrificed by excessive anesthesia. All efforts were made to minimize the number of mice used and to decrease their suffering. Finally, the uterus and embryos from each mouse were isolated. Routine HE staining was performed on the mouse uterus.

Ethics approval and consent to participate

The Institutional Review Board of the University of Chinese Academy of Sciences Shenzhen Hospital approved the protocol used in the present study (Approval No. KY-2018-040) and all procedures were performed in accordance with the ethical standards established in the Declaration of Helsinki. All patients provided written informed consent before

commencing the present study. The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Chinese Academy of Sciences Shenzhen Hospital (Guangming) (Approval No. KY-2018-040).

Flow cytometry

Heparinized venous blood of the participants or mice was dispensed into two tubes and the blood was then incubated with 2 mL of red blood cell lysis buffer (#C3702; Beyotime, Shanghai, China) in the dark at room temperature for 10 minutes. Following centrifugation at 2000 ×g at 4°C for 10 minutes, samples were washed twice with phosphate-buffered saline (PBS). Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (cat. no. 553046, 1:200; BD Biosciences, Franklin, NJ, USA), allophycocyanin-conjugated anti-CD25 (cat. no. 557192, 1:200; BD Biosciences), and phycoerythrin-conjugated anti-Foxp3 (cat. no. 563101, 1:200; BD Biosciences) antibodies were added to one tube. Additionally, FITC-conjugated immunoglobulin G (IgG) (cat. no. 555988, 1:5), allophycocyanin-conjugated IgG (cat. no. 550931, 1:5), and phycoerythrin-conjugated IgG (cat. no. 560951, 1:5) antibodies (BD Biosciences) were added to the other tube as a control. After 20 minutes of incubation at room temperature in the dark, the samples were washed twice with PBS and resuspended in 500 μL of cell staining buffer. Finally, the cells were analyzed on a FACSCalibur flow cytometer (Accuri C6; BD Biosciences).

Total RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from 2 mL of peripheral blood of the participants or mice by using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). A cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) was used for synthesis of cDNA according to the manufacturer's protocol. Quantitative reverse transcription-polymerase chain reaction was performed to detect mRNA expression levels using SYBR Green and a LightCycler 480 detection system (Roche Diagnostics, Shanghai, China), and the reaction volume was 20 μL. Glyceride-3-phosphate dehydrogenase mRNA levels were used for normalization. The thermocycling conditions were as follows: pre-denaturation at 95°C for 3.5 minutes, followed by 38 cycles of 90°C (15 s) and 60°C (30 s). The validity of the analysis was evaluated by melting curve analysis and quantitative reverse transcription-polymerase chain reaction results were quantified using the $2^{-\Delta\Delta Cq}$ method.[19]

Enzyme-linked immunosorbent assay

Peripheral blood of the participants or mice was centrifuged at 10,000 ×g at 4°C for 10 minutes and serum was used to measure concentrations of interferon (IFN)-γ (MIF00), interleukin (IL)-2 (#M2000), IL-4 (#M4000B), and IL-10 (M1000B). The detection kits were purchased from R&D (Minneapolis, MN, USA).

Immunohistochemistry

Uterine villi or uterine tissue from mice were fixed in 4% paraformaldehyde in PBS at room temperature for 15 minutes and cut into 3-μm sections. The sections were stained immunohistochemically with antibodies against Foxp3 (cat. no. ab99963; Abcam, Cambridge, UK) at a 1:100 dilution. The slides were incubated with the primary antibody for 2 hours at 37°C. Goat anti-rabbit IgG peroxidase-conjugated secondary antibody (cat. no. ab6721; Abcam) was used with 3,3-

diamino-benzidine, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as an enzyme substrate. Mean optical density was calculated using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

Data were statistically analyzed and graphed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). All results are presented as the mean ± standard deviation. Multiple comparisons were made among ≥three groups using one-way analysis of variance followed by the Bonferroni post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. We did not perform a sample size calculation. Therefore, the limited number of samples may have affected the statistical significance of the results.

Results

Subjects

The URSA group was composed of 45 women who had a mean age of 29.6 ± 2.6 years and had at least three successive miscarriages with unexplained etiology. The control group was composed of 40 women who had a mean age of 28.28 ± 2.75 years and had normal pregnancies and successful delivery.

Proportion of $CD4^+CD25^+Foxp3^+$ Tregs is decreased and the inflammatory response is increased in women with URSA

The proportion of $CD4^+CD25^+Foxp3^+$ Tregs in the URSA group was significantly lower ($5.1\% \pm 0.82\%$) than that in the control group ($7.8\% \pm 0.76\%$, $P < 0.05$). Additionally, Foxp3 mRNA expression levels were significantly lower in the URSA group than in the control group ($P < 0.05$; Figure 1). The ratios of IFN-γ/IL-4 and IL-2/IL-10 and TLR4 and NF-κB mRNA expression levels in peripheral blood in the URSA group were significantly higher than those in the control group (all $P < 0.05$). Moreover, HE staining and immunohistochemistry showed that the URSA group had a more turbid nucleoplasm and disorderly arrangement of villi, with significantly lower Foxp3 protein expression levels compared with the control group ($P < 0.05$; Figure 2).

$CD4^+CD25^+Foxp3^+$ Tregs affect the abortion rate in mice

Isolated embryos from the mice were observed and the embryo absorption rate was calculated (Table 1). Mouse embryos appear as strings of beads, which were found in all mice in each group. However, the morphology of embryos varied among the different groups. Normally developing embryos appeared red with intact amniotic sacs and a placenta, and were shaped like embryos. Absorbed embryos were small without placental formation and were partially visible as dark red or even brown clots. The embryo absorption rate was significantly higher in the model group compared with the control group ($P < 0.05$), which indicated that the model was successfully constructed. TLR4 antagonist E5564 treatment significantly reduced the embryo absorption rate and treatment with the TLR4 agonist LPS resulted in a higher embryo absorption rate compared with that in the sham group (both $P < 0.05$).

Proportion of $CD4^+CD25^+Foxp3^+$ Tregs is decreased in abortion-prone model mice

In the abortion-prone model (Figure 3), the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was significantly lower compared with that in the control group ($P < 0.05$). When mice

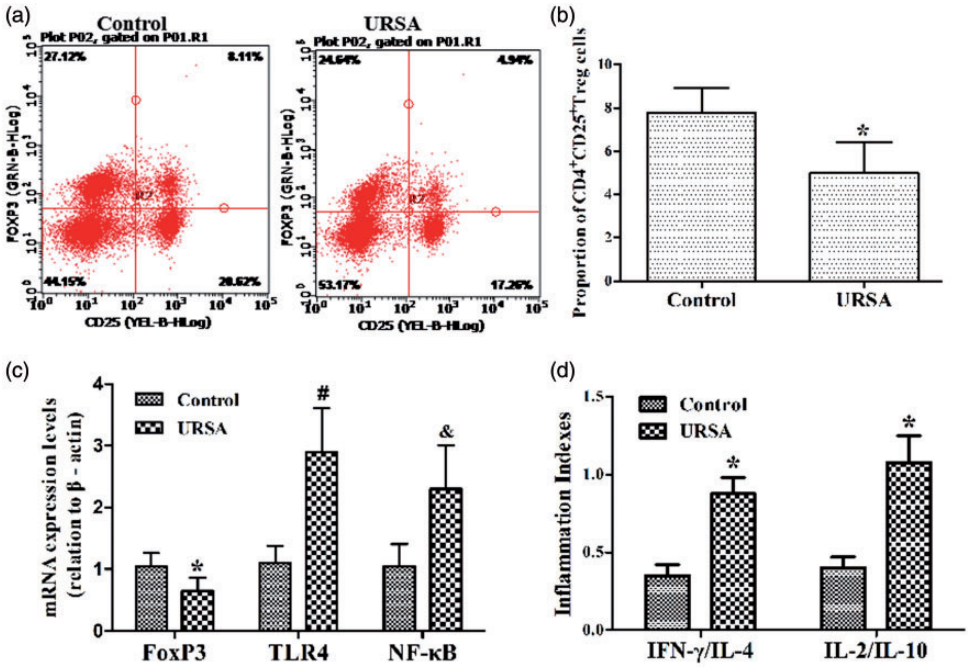

Figure 1. Proportion of $CD4^+CD25^+Foxp3^+$ Tregs and the inflammatory response in women with URSA. (a) $CD4^+CD25^+Foxp3^+$ regulatory T cells measured in peripheral blood using flow cytometry with fluorescein isothiocyanate-conjugated anti-CD4, allophycocyanin-conjugated anti-CD25, and phycoerythrin-conjugated anti-Foxp3 antibodies. (b) The proportion of $CD4^+CD25^+Foxp3^+$ Tregs in the control and URSA groups. (c) Foxp3, TLR4, and NF-κB mRNA expression levels in the peripheral blood as measured by quantitative reverse transcription-polymerase chain reaction. (d) Inflammation indices of IFN-γ/IL-4 and IL-2/IL-10 in the peripheral blood as measured by enzyme-linked immunosorbent assay. Data are shown as the mean ± standard deviation (n = 40 for the control group and n = 45 for the URSA group). *$P < 0.05$, #$P < 0.05$, and &$P < 0.05$ vs. the corresponding control group
CD, cluster of differentiation; Foxp3, forkhead box p3; Tregs: regulatory T cells; URSA: unexplained recurrent spontaneous abortion; TLR4: toll-like receptor 4; NF-κB, nuclear factor-κB; IFN-γ, interferon-γ; IL, interleukin.

were treated with E5564, the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was significantly higher compared with that in the sham group ($P < 0.05$). When mice were treated with LPS, the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was significantly lower compared with that in the sham group ($P < 0.05$).

$CD4^+CD25^+Foxp3^+$ Tregs affect the inflammatory response of abortion-prone mice

In the peripheral blood of the abortion-prone model, Foxp3 mRNA expression levels were downregulated compared with the control group ($P < 0.05$), which corresponded to downregulation of the proportion of $CD4^+CD25^+Foxp3^+$ Tregs mentioned above. Additionally, TLR4 and NF-κB mRNA expression levels in the model group were upregulated compared with those in the control group (both $P < 0.05$). When mice were treated with E5564, TLR4 and NF-κB mRNA expression levels were downregulated, and LPS upregulated TLR4 and NF-κB mRNA expression levels compared with the sham group (all $P < 0.05$; Figure 4a). The ratios

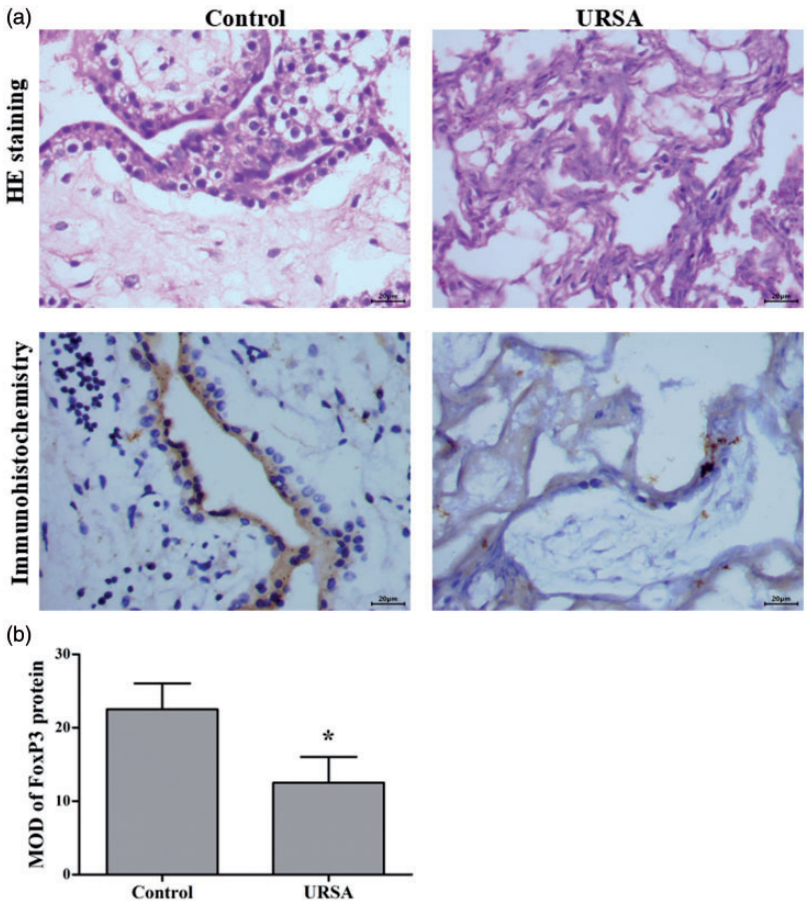

Figure 2. HE staining and Foxp3 immunohistochemical staining of uterine villi. (a) Images of HE staining and Foxp3 immunohistochemical staining of the groups. Magnification, 400×. (b) MOD of Foxp3 in the control and URSA groups. Data are shown as the mean ± standard deviation (control group, n = 10 and URSA group, n = 8). *$P < 0.05$ vs. the control group
HE, hematoxylin and eosin; Foxp3, forkhead box p3; MOD, mean optical density; URSA, unexplained recurrent spontaneous abortion.

of IFN-γ/IL-4 and IL-2/IL-10 were significantly higher in the abortion-prone model group and lower in the E5564 group compared with the sham group (all $P < 0.05$; Figure 4b).

HE staining showed that abortion-prone model mice showed less endometrial thickness, greater inflammatory cell infiltration, and lower gland numbers compared with the control group (Figure 5). E5564 caused reduced inflammatory cell infiltration, greater endometrial thickness, and a higher number of glands compared with the sham group. However, LPS induced inflammatory cell infiltration and resulted in less endometrial thickness and a lower number of glands compared with the sham group. In the uterus, immunohistochemical results were similar to mRNA expression in peripheral blood. In the E5564 group, Foxp3 protein expression was significantly higher than that in

Table 1. Embryo absorption in abortion-prone mice

Groups	n	Total number of embryos	Number of absorbed embryos	Absorption rate (%)
Control	10	85	6	7.06
Model	10	87	20	22.99*
Sham	10	84	21	25.00
E5564	10	86	12	13.95#
LPS	10	85	26	30.59&

*$P < 0.05$ vs. the control group; #$P < 0.05$ and &$P < 0.05$ vs. the sham group.
LPS, lipopolysaccharide.

the sham group ($P < 0.05$). Additionally, Foxp3 protein expression was significantly lower in the LPS group than in the sham group ($P < 0.05$; Figure 6).

Discussion

In clinical practice, balance of Th1/Th2 cells and increased Tregs can be beneficial for pregnancy.[17] T cell activity tends to be Th2 immunity at the fetomaternal interface, which helps protect the pregnancy, while Th2 immunity is disrupted in recurrent

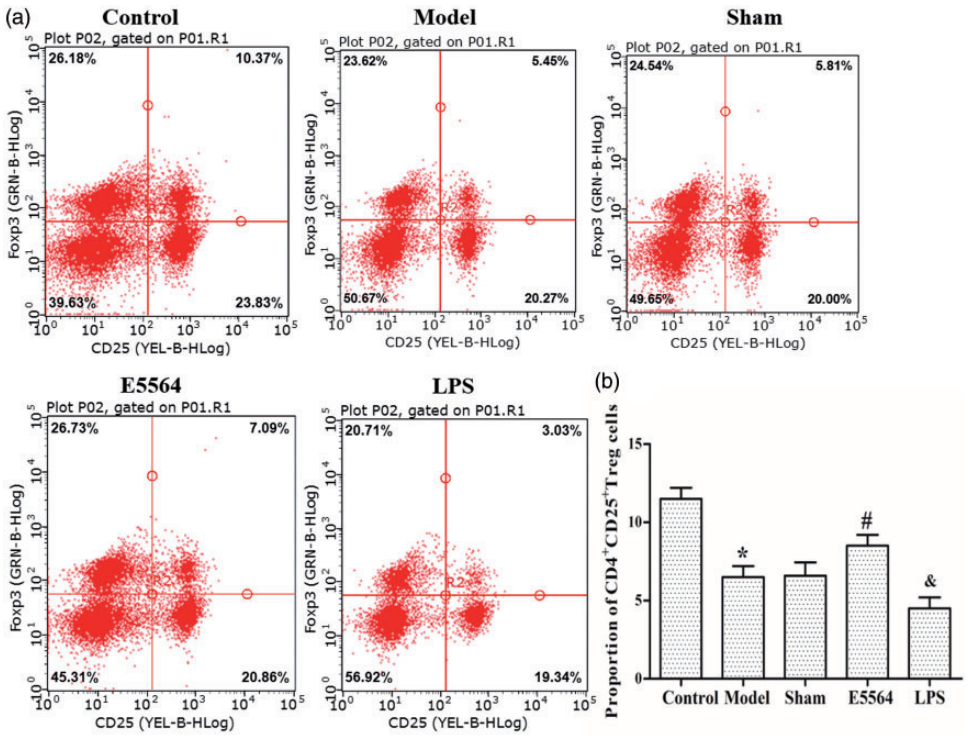

Figure 3. Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ Tregs in mice. (a) $CD4^+$ and $CD25^+$ Tregs measured using flow cytometry with fluorescein isothiocyanate-conjugated anti-CD4, allophycocyanin-conjugated anti-CD25, and phycoerythrin-conjugated anti-Foxp3 antibodies. (b) The proportion of $CD4^+CD25^+Foxp3^+$ Tregs in each group. Data are shown as the mean ± standard deviation (n = 10 in each group). *$P < 0.05$ vs. the control group; #$P < 0.05$ and &$P < 0.05$ vs. the sham group
CD, cluster of differentiation; Foxp3, forkhead box p3; Tregs, regulatory T cells; LPS, lipopolysaccharide.

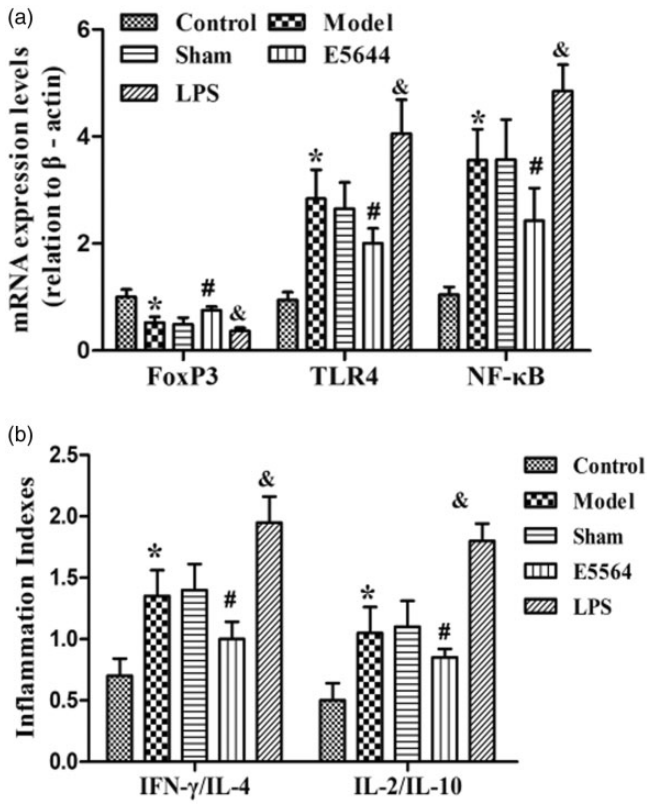

Figure 4. Inflammatory response of mice in each group. (a) Foxp3, TLR4, and NF-κB mRNA expression levels in the peripheral blood as measured by quantitative reverse transcription-polymerase chain reaction. (b) Inflammation indices of IFN-γ/IL-4 and IL-2/IL-10 in peripheral blood as measured by enzyme-linked immunosorbent assay. Data are shown as the mean $\pm$ standard deviation (n=10 in each group). *$P < 0.05$ vs. the control group; $^{\#}P < 0.05$ and $^{\&}P < 0.05$ vs. the sham group
Foxp3, forkhead box p3; TLR4, toll-like receptor 4; NF-κB, nuclear factor-κB; IFN-γ, interferon-γ; IL, interleukin.

abortion.[20] $CD4^+CD25^+$ Tregs have immunoregulatory functions and can modulate Th1 activity in early human pregnancy.[21] Yang *et al.*[22] found that allogeneic lymphocyte therapy enhanced the number of $CD4^+CD25^+$ Tregs in peripheral blood and that the proportion of $CD4^+CD25^+$ Tregs may serve as a biomarker for monitoring allogeneic lymphocyte therapy in patients with URSA. Cyclosporin A is a powerful immunosuppressor that is widely used to prevent organ rejection and to treat certain autoimmune diseases in the clinic.[23] Du *et al.*[24] found that cyclosporin A improved the pregnancy outcome and induced fetomaternal immune tolerance by upregulating the proportion of peripheral $CD4^+CD25^+Foxp3^+$ Tregs in mice.[7] The present study also showed that the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was decreased in patients with URSA and in abortion-prone model mice. Additionally,

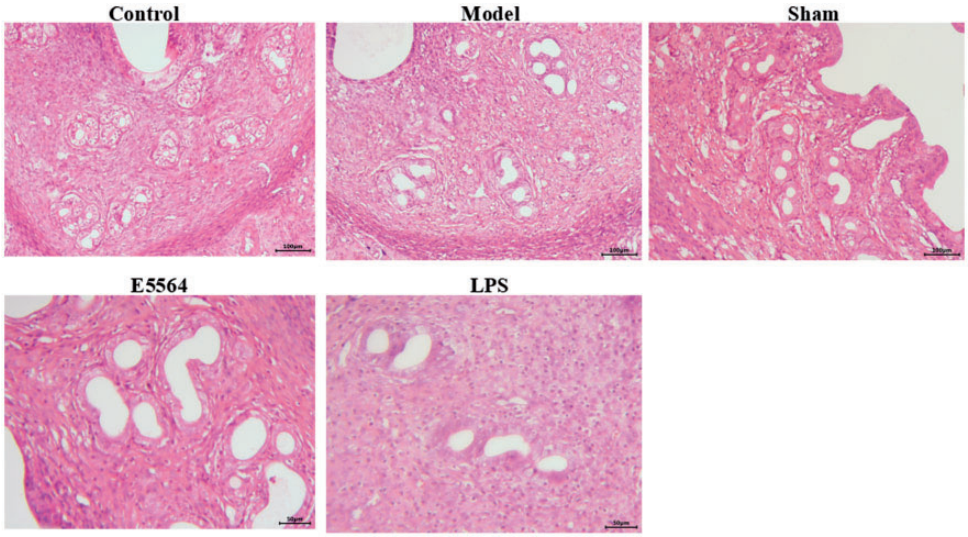

Figure 5. Images of hematoxylin and eosin staining of mouse uterine tissue in each group. Magnification, 100×
LPS, lipopolysaccharide.

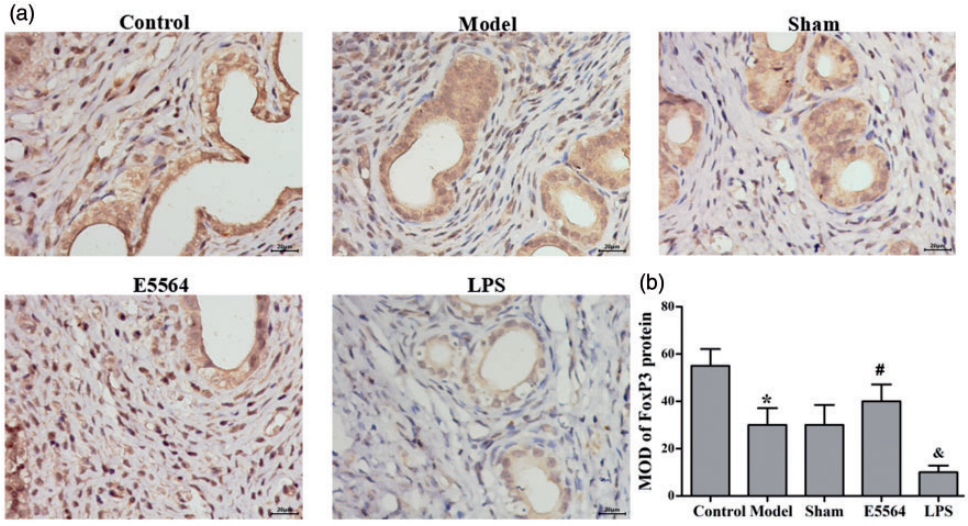

Figure 6. Foxp3 protein expression in mouse uterine tissue. (a) Images of foxp3 immunohistochemical staining of mouse uterine tissue in each group. Magnification, 400×. (b) MOD of Foxp3 in each group. Data are shown as the mean ± standard deviation (n = 10). *$P < 0.05$ vs. the control group; $^{\#}P < 0.05$ and $^{\&}P < 0.05$ vs. the sham group
Foxp3, forkhead box p3; MOD, mean optical density; LPS, lipopolysaccharide.

when the proportion of $CD4^+CD25^+$ $Foxp3^+$ Tregs increased after E5564 treatment, the embryo absorption rate decreased in mice. In contrast, when the proportion of $CD4^+CD25^+Foxp3^+$ Tregs decreased after LPS treatment, the embryo absorption rate increased. These data indicate that the proportion of $CD4^+CD25^+Foxp3^+$ Tregs may be used as a specific biomarker for URSA and that the TLR4/NF-κB pathway is involved in $CD4^+CD25^+Foxp3^+$ Treg-mediated pregnancy or abortion.

TLR4 is expressed in Tregs and Tregs are characterized as $CD4^+CD25^+$ $Foxp3^+$.[15] Foxp3 protein is considered to be the most reliable molecular marker of mature Tregs and is involved in the development and function of Tregs.[24] The present study showed that TLR4 and Foxp3 mRNA expression was decreased in patients with URSA and in abortion-prone model mice, with a decreased proportion of $CD4^+CD25^+Foxp3^+$ Tregs. Taken together, previous findings[12–15,24] and the present study suggest that inhibiting the TLR4/NF-κB signaling pathway of $CD4^+$ $CD25^+Foxp3^+$ Tregs has potential therapeutic advantages for URSA. To verify this hypothesis, the TLR4 antagonist E5564 and the TLR4 agonist LPS were used in our study. Following treatment with E5564, TLR4 and NF-κB mRNA expression levels were decreased, which in turn increased expression of Foxp3 mRNA/protein and the proportion of $CD4^+CD25^+$ $Foxp3^+$ Tregs. Following treatment with LPS, TLR4 and NF-κB mRNA expression was increased, which in turn decreased Foxp3 mRNA/protein expression and the proportion of $CD4^+CD25^+Foxp3^+$ Tregs. These data indicate that $CD4^+CD25^+$ $Foxp3^+$ Tregs may regulate the TLR4/NF-κB pathway and this pathway may regulate the proportion of $CD4^+CD25^+$ $Foxp3^+$ Tregs.

Regulation of Th1/Th2 immune balance is regarded as an important mechanism determining the survival of the fetus in the uterus in humans and other mammals.[25–27] Several mechanisms of Treg-mediated suppression of the immune response have been proposed, including secretion of immunosuppressive cytokines, cell contact-dependent suppression, and functional modification or killing of antigen-presenting cells.[28,29] Production of Th2-type cytokines, including IL-10 and IL-4, favors maintenance of mammalian pregnancy, while Th1-type cytokines, including IL-2 and IFN-γ, mediate fetal rejection.[30] The ratios of IFN-γ/IL-4 and IL-2/IL-10 can reflect the cytokine balance. The inflammatory response is stronger when the cytokine ratio is high and the environment is closer to immune balance when this ratio is low. The present study showed that the ratios of IFN-γ/IL-4 and IL-2/IL-10 were increased in patients with URSA and abortion-prone model mice, which suggested that the immune balance was disrupted in URSA. Additionally, E5564 treatment decreased the ratios of IFN-γ/IL-4 and IL-2/IL-10, and LPS increased these ratios. HE staining also confirmed the above-mentioned results. These results suggest that $CD4^+CD25^+Foxp3^+$ Tregs affect inflammatory cytokine release in mice with URSA via the TLR4/NF-κB pathway.

Conclusion

$CD4^+CD25^+Foxp3^+$ Tregs regulate the expression and release of inflammatory cytokines via the TLR4/NF-κB pathway. In contrast, inflammatory cytokine levels have a feedback effect on the proportion of $CD4^+CD25^+Foxp3^+$ Tregs, thereby promoting immune balance in the body. The TLR4 antagonist E5564, similar to cyclosporin A, promotes immune homeostasis by modulating $CD4^+CD25^+Foxp3^+$ Tregs and inflammatory cytokines, and may be a novel and potential drug for treating URSA. Regrettably, there is a lack of

research on clinical applications and systematic mechanisms of E5564 in URSA. Therefore, further research is required to study the other effects of E5564 on URSA and other underlying mechanisms.

Availability of data and materials

The datasets used and/or analyzed in the current study appear in the submitted article.

Declaration of conflicting interest

The author(s) declare that there is no conflict of interest.

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