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Full length article

Expression of TLR4 and its effect on Treg cells in early pregnancy decidual stromal cells after lipopolysaccharide treating

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ABSTRACT

Objective: To investigate the expression of TLR4 in human early pregnancy decidual stromal cells (DSCs) induced by lipopolysaccharide (LPS) and its effect on the peripheral blood regulatory T (Treg) cells subgroup in women of childbearing age.

Method: Isolating and cultivating normal human early pregnancy DSCs followed by treatment with 0, 25, 50, 100 and 200 ng/ml LPS, and the expression level of TLR4 mRNA was detected by RT-PCR. After 3 or 4 generation we divide the DSCs into 5 groups: ①Control group: Cultivation of peripheral blood lymphocyte (PBLC); ②Co-cultivation group: Co-cultivation of PBLC and DSCs; ③LPS stimulation group: PBLC + DSCs + LPS; ④PDTC blocking-up group: PBLC + DSCs + LPS + PDTC; ⑤TLR4 blocking-up group: PBLC + DSCs + LPS + TLR4mAb. In ①–④ groups, western blot was used to detect the expression of inhibitory factor-κB (IκB-α) protein and RT-PCR was used to detect the expression of FoxP3 mRNA. In ①–⑤ groups, flow cytometry was applied to detect the percentage of Treg cells subgroup.

Results: The purity of primary cultured DSCs was more than 95%. RT-PCR results showed that the expression level of TLR4 mRNA increased gradually with the augment of LPS concentration. Western blot and RT-PCR showed that the expression of IκBα protein and FoxP3 mRNA in the other 3 groups was significantly higher than that in the control group ($P < 0.05$), and the expression of IκBα protein and FoxP3 mRNA in LPS stimulation group was lower than that in the co-cultivation group ($P < 0.05$). Compared with the LPS stimulation group, the expression of IκBα protein and FoxP3 mRNA in PDTC blocking-up group was higher than that in the LPS stimulation group ($P < 0.05$), but still lower than the co-cultivation group ($P < 0.05$). The proportion of Treg cells in the other 4 groups detected by flow cytometry was significantly higher than that in the control group ($P < 0.05$). Compared with the co-cultivation group, the Treg cells ratio of the LPS stimulation group was significantly decreased ($P < 0.05$). The proportions of Treg cells in PDTC blocking-up group and TLR4 blocking-up group were higher than that in the LPS stimulation group, but still lower than that in the co-cultivation group ($P < 0.05$). There was no significant difference between the PDTC blocking-up group and the TLR4 blocking-up group ($P > 0.05$).

Conclusion: Human early pregnancy DSCs can promote the differentiation of Treg cells. LPS can stimulate the expression of TLR4 in early pregnancy DSCs and decrease the proportion of Treg cells in PBLC, with NF-κB signaling pathway being the potential underlying mechanisms.

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Introduction

In normal pregnancy, there are adaptive immune changes in the maternal-fetal interface, which drive the mother body immunologically tolerant to semi-homologous transplantation of embryos. At the same time, the immune barrier of maternal-fetal interface has the function of maintaining the host defense response and preventing the pregnancy tissues from pathogenic microorganisms damage. A large number of studies have shown that intrauterine infection is an important cause of miscarriage, premature birth, fetal intrauterine growth restriction, preeclampsia and other diseases of pregnancy [1,2].

It has been known that bacterial infections or bacterial components such as lipopolysaccharide(LPS) can cause abortion by inducing proinflammatory molecules [3,4]. Toll-like receptor (TLRs) family is an important immune receptor of the organism, in which TLR4 is the first member of TLRs found in Homo sapiens [5]. TLR4 can not only mediate innate immunity, but also mediate immune response [6], so it functions as a bridge linking natural immunity and acquired immunity of the organism. Regulatory T

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(Treg) cells are novel lymphocyte subgroups discovered in recent years, which are closely related to the development of immune tolerance diseases, having become a hotspot of immunology research in recent years [7,8]. It has been reported that patients with recurrent spontaneous abortion may have changes of both TLR4 expression and Treg cells [9–11]. However, the relationship between TLR4 and Treg cells in maternal-fetal interface at early pregnancy is yet unclear. Frederic Schatz [12] found that TLR4 expression in DSCs was significantly higher than that in invasive trophoblast cells, suggesting that DSCs might be the primary target for TLR4 to recognize invasive G-bacteria and inflammation-related hazards.

We co-cultivated human early pregnancy DSCs and PBLC in women of childbearing age. Then intervened them with LPS and atypical inhibitor of NF-κB (PDTC) to observe the effects of DSCs on Treg cells subgroup in early pregnancy and explore the potential mechanisms.

Materials and methods

Materials

The specimens were collected from 11 cases of normal early pregnancy female outpatient decidual tissues undergoing voluntary abortion in the Affiliated Hospital of Xuzhou Medical University. The mean age of patients was (27.2 ± 5.9) years, 6–8 weeks pregnant, without clinical symptoms such as threatened abortion, and they were all confirmed by B-ultrasound with single pregnancy birth without any infection, spontaneous abortion or other past medical history. Under the informed consent of patients, tissues from abortion were collected aseptically into sterile saline (containing 400 U/mL penicillin and 100 U/mL streptomycin) and then were sent to the laboratory within 15 min. Peripheral blood lymphocytes were collected from female volunteers of reproductive age. This study was approved by the Medical Ethics Committee of the Affiliated Hospital of Xuzhou Medical University.

The chief reagents are as follows: LPS (Sigma, USA), collagenase I (Sigma), hyaluronidase (Sigma), DMEM/F12 medium (Hyclone, USA), fetal bovine serum (Sijiqing, Hangzhou, China), rabbit anti-human PRL Antibody (Bioss, Beijing, China), lymphocyte separation fluid (LSM, MP biomedicals company, USA), PDTC (Beyotime, Shanghai, China), rabbit anti-human TLR4 antibody (Novus biologicals company, USA); Anti-human IκB-α antibody (Cell signaling technology, USA), CD4 and CD25 antibody (BD biosciences, USA), Trizol (Invitrogen, USA), reverse transcription polymerase chain reaction (RT-PCR) kit (Tiangen biotech company, Beijing, China), polymerase chain reaction kit 2×Taq Master Mix (Tiangen), all PCR primers were synthesized and purchased from Shanghai Sangon Biotech Company.

Isolation of PBLC: We extracted 10 ml peripheral blood from healthy women volunteers (heparin anticoagulant). PBLC was isolated from human lymphocyte separation solution and suspended in RPMI1640 medium (containing 10% fetal bovine serum) with the cell density of 1×10^6/ml.

Methods

Isolation and cultivation of early pregnancy DSCs

The cultivation methods referred to the reference [13], sterile collection of abortive decidual tissues were placed in sterile saline containing Penicillin-Streptomycin antibiotics followed by rinsing adequately to remove the villi and red blood cells; the tissues were cut into pieces of $1 \times 1 mm^3$ size and mixed in 1 ml complex digestive enzyme followed by 37 °C shaker digestion for 30 min; add equal volume of the culture medium to halt digestion, then wash the centrifugal cells twice by the culture medium; pipe the cells up and down into suspension and transfer them into a 10 cm culture dish. The dish were then incubated at 37 °C in a 5% CO_2 incubator and replaced with fresh culture medium after 48 h.

Experimental groups

The DSCs cultivated to the third or fourth generation were divided into 5 groups. ①Control group: Cultivation of PBLC; ②Co-cultivation of PBLC and DSCs group; ③LPS stimulation group: PBLC + DSCs + LPS; ④PDTC blocking-up group: PBLC + DSCs + LPS + PDTC; ⑤TLR4 blocking-up group: PBLC + DSCs + LPS + TLR4mAb. The ratio of DSCs and PBLC is 1:10, the concentration of LPS is 100 ng/ml [14], the concentration of PDTC is 30 μmol/L, the concentration of TLR4mAb is 1 μg/ml [15]. All groups of cells were cultivated in 37 °C, 5% CO_2 incubator for 5 days, and then we collected PBLC. In①∼④groups, RT-PCR was used to detect the transcription level of FoxP3, western blot was used to detect the expression of IκB-α protein. In①∼⑤groups, flow cytometry was applied to detect the proportion of Treg cells subgroup.The DSCs were treated with 0, 25, 50, 100, 200 ng/ml LPS for 24 h, and the levels of TLR4 mRNA were detected by RT-PCR.

Identification of cultivated DSCs by immunofluorescence assay

Take the third generation of climbing tablets DSCs and fix them with 4% paraformaldehyde for 15 min. Then we use 0.1% Triton X-100 to rupture the cell membranes for 10 min followed with 5% BSA blocking for 1 h. Next, we add appropriate amount of PRL antibody (1:50) at 4 °C overnight. The next day, we add 1:300 fluorescently labeled secondary antibody and incubate for 1 h at room temperature followed by reaction with DAPI for 90 s. Fluorescence staining of DSCs was observed through a fluorescence microscope of 200×magnification.

RT-PCR to detect the levels of TLR4 mRNA and FoxP3 mRNA in PBLC

The co-cultivated PBLC were collected and the total RNA was extracted by Trizol reagent. Measure RNA concentration and purity, and then we proceed reverse transcription to synthesize cDNA. The PCR reaction system are as follows: 2 μl of cDNA, 1 μl of upstream and downstream primers, 12.5 μl of 2×TaqMix, 8.5 μl ofribozyme free water to form 25 μl reaction system. The primer sequences of TLR4: upstream: 5′-AAGCCGAAAGGTGATTGTTG-3′, downstream: 5′-CTGTCCTCCCACTCCAGGTA-3′ (213bp); the primer sequences of FoxP3: upstream: 5′-TCCCAGAGTTCCTCCACAAC-3′, downstream: 5′-ATTGAGTGTCCGCTGCTTCT-3′ (122bp); the primer sequences of β-actin: upstream: 5′-CGGGAAATCGTGCGTGACAT-3′, downstream: 5′-CGGACTCGTCATACTCCTGCTTG-3′ (481bp). The reaction conditions are as follows: 94°C pre-denaturation for 5 min, then 94 °C for 30 s, 59 °C for 30 s, 72 °C for 60 s, for 35 cycles, and then 72 °C extension for 10 min. The PCR products were isolated by 2% agarose gel electrophoresis. The ratio of absorbance (OD) of each target gene and β-actin product was calculated and the OD ratio was used as the relative quantitative expression level of mRNA.

Western blot to detect the expression of IκB-α protein in PBLC

The total protein was extracted from PBLC and the concentration of protein was determined by BCA method. 50 μg of protein was taken from each group for SDS-PAGE gel electrophoresis, and then was transferred to the PVDF membrane followed by 2 h blocking. 1:1000 IκBα and 1:1000 β-actin primary antibody were used for incubation at 4°C overnight. And the next day, we added 1:1000 secondary antibody for incubation at room temperature for 2 h. Then, we used chemiluminescence analyzer to take pictures. The relative gray level of each band relative to β-actin was used as the relative expression level of the protein.

Flow cytometry to detect the proportion of Treg cells

The co-cultivated PBLC were collected and divided into control tube and experimental tube. 10 μl of CD4-FITC and CD25-PE were added and incubated in the dark place at room temperature for 30 min. Washed and discarded the supernatant. After that, the cells were resuspended in 300 μl of wash solution and detected by flow cytometry. $CD4^+$ cells were distinguished by CD4 histogram, and the position of Mark was adjusted by the same type of control tube. Under the same experimental conditions, the proportion of $CD4^+CD25^+$Treg/$CD4^+$T cells were measured and the data were achieved and analyzed by CellQuest software.

Statistical analysis

The data were analyzed by SPSS16.0 software. The grouped data were shown as mean ± standard deviation ($\bar{x} \pm s$). Single factor analysis of variance was used to compare the results of the two groups. The difference was statistically significant with $P < 0.05$.

Results

Morphological characteristics of DSCs

Normal DSCs are spindle-shaped or polygonal with rich cytoplasm, and their nucleolus are oval and centered with slightly lighter blue staining and the nucleolus are large and obvious, as shown in Fig. 1A, B.

Identification of DSCs by immunofluorescence assay

DSCs have the characteristics of synthesizing placenta prolactin (PRL), which is one of its functional features. In this study, immunofluorescence assay was used to detect the expression of PRL in decidual cells as a way to identify DSCs in vitro. Through the microscope we found the PRL in DSCs was located in the cytoplasm and expressed as green fluorescence, additionally, the nearer to the nucleus, the deeper the staining was. The nucleus expressed as blue fluorescence and 95% of the cells were green fluorescence-positive, as shown in Fig. 1C.

Effects of different concentrations of LPS on TLR4 mRNA expression in DSCs

The results of RT-PCR showed that the expression of TLR4 increased along with raised concentration of LPS after treated with different concentrations of LPS, and the difference was statistically significant ($P < 0.05$), as shown in Fig. 2.

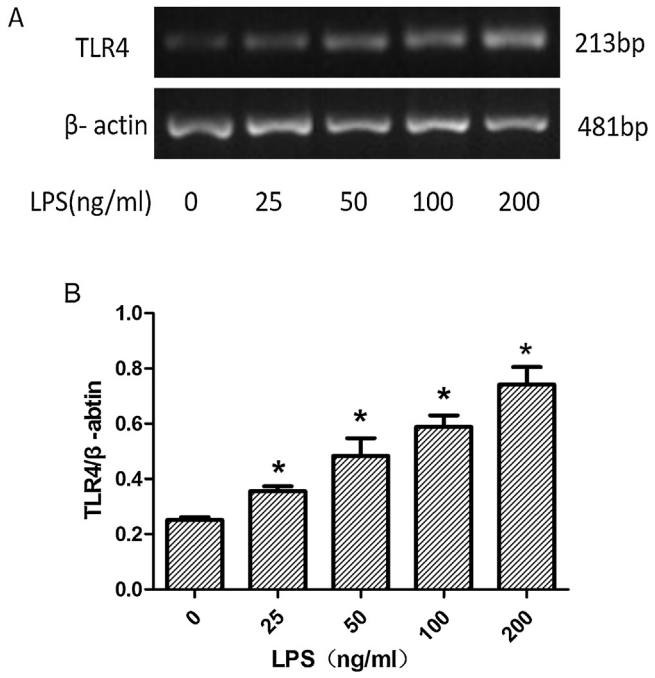


Fig. 2. Expression of TLR4 mRNA in different concentrations of LPS.

Effect of DSCs on the expression of IκBα protein

The expression of IκBα protein in co-cultivation group was significantly higher than that in the other three groups ($P < 0.05$), suggesting that DSCs could promote the expression of IκBα protein. After LPS stimulation, IκBα protein expression was significantly lower than the co-cultivation group. IκBα protein expression was increased after we added PDTC to block-up NF-κB signaling pathway, but still lower than that of co-cultivation group, and the difference was statistically significant ($P < 0.05$). As shown in Fig. 3 and Table 1.

Effect of DSCs on the expression of FoxP3 mRNA

The expression of FoxP3 mRNA in co-cultivation group was significantly higher than that in the other 3 groups ($P < 0.05$), suggesting that DSCs could promote the expression of FoxP3 mRNA. After LPS stimulation, FoxP3 mRNA expression was significantly lower than the co-cultivation group. FoxP3 mRNA expression was raised after we added PDTC to block-up NF-κB signaling pathway, but still lower than that of co-cultivation group,

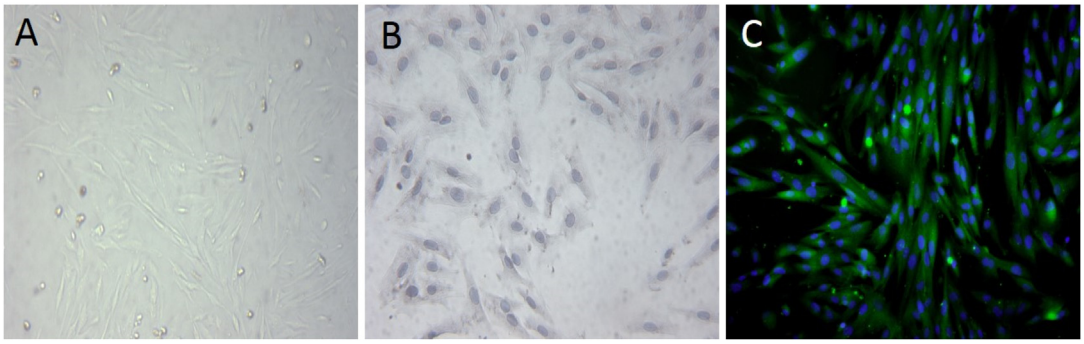


Fig. 1. Morphology and PRL expression in decidual cells (×200 magnification). A. Morphology under the light microscope; B. HE staining; C. The expression of PRL in the decidual cells via immunofluorescence assay.

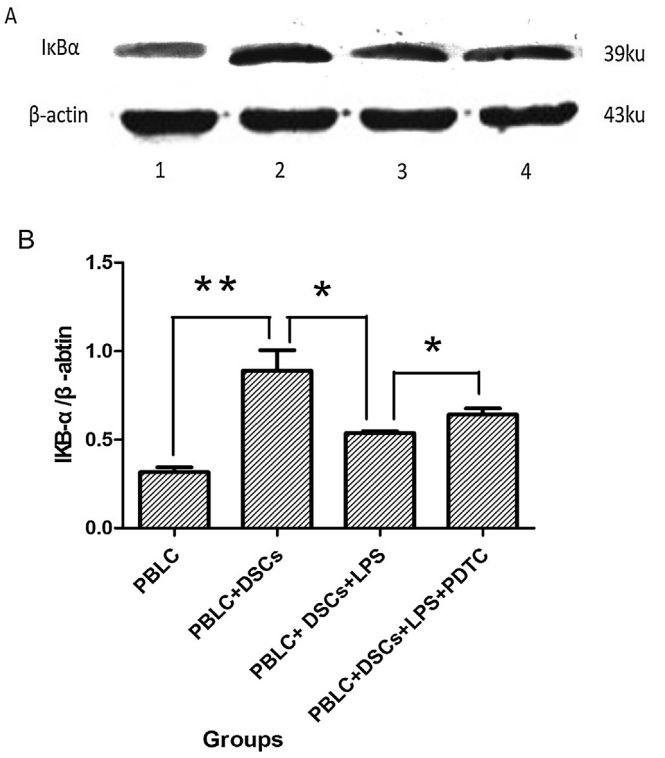


Fig. 3. Expression of IκBα protein in different cultivation groups. 1. Control group; 2. DSCs + PBLC; 3. DSCs + PBLC + LPS; 4. DSCs + PBLC + LPS + PDTC.

Table 1
Effects of DSCs on expression of IκB-α protein and FoxP3 mRNA ($x(\_)\pm\ s$).

Groups	IκB-α protien	FoxP3 mRNA
Control group	$0.317 \pm 0.047^{*}$	$0.295 \pm 0.231^{\#}$
Co-cultivation group	$0.890 \pm 0.200^{*}$	$0.603 \pm 0.047^{\#}$
LPS stimulation group	$0.537 \pm 0.020^{*}$	$0.412 \pm 0.032^{\#}$
PDTC blocking-up group	$0.642 \pm 0.060^{*}$	$0.492 \pm 0.044^{\#}$

Comparison between groups, $^{*}P$ <0.05, $^{\#}P$ <0.05.

and the difference was statistically significant ($P < 0.05$). As shown in Fig. 4 and Table 1.

Effects of DSCs on peripheral blood Treg cells subgroup

Compared with the control group, the proportion of CD4+CD25+/CD4 + T cells in the co-cultivation group increased significantly ($P < 0.05$), suggesting that DSCs could promote the differentiation of Treg cells. Compared with the co-cultivation group, the proportion of $CD4^{+}CD25^{+}/CD4^{+}$T cells in LPS-stimulation group was significantly reduced ($P < 0.05$), and the reduced degree was less than that in LPS stimulation group; compared with LPS stimulation group, the proportion of $CD4^{+}CD25^{+}/CD4^{+}$T cells increased in both TLR4 blocking-up group and PDTC blocking-up group, but there was no significant difference between these two group ($P > 0.05$). It suggests that DSCs can promote the proliferation of Treg cells and activation of TLR4 signaling pathway can inhibit the effect of DSCs in promoting differentiation of Treg cells, as shown in Fig. 5 and Table 2.

Discussion

Treg cells are subgroups possessing immunosuppressive functions found in recent years. As the specific transcription factor, FoxP3 can regulate cell proliferation of Treg cells. Treg cell-mediated immunosuppression plays a key role in autoimmune

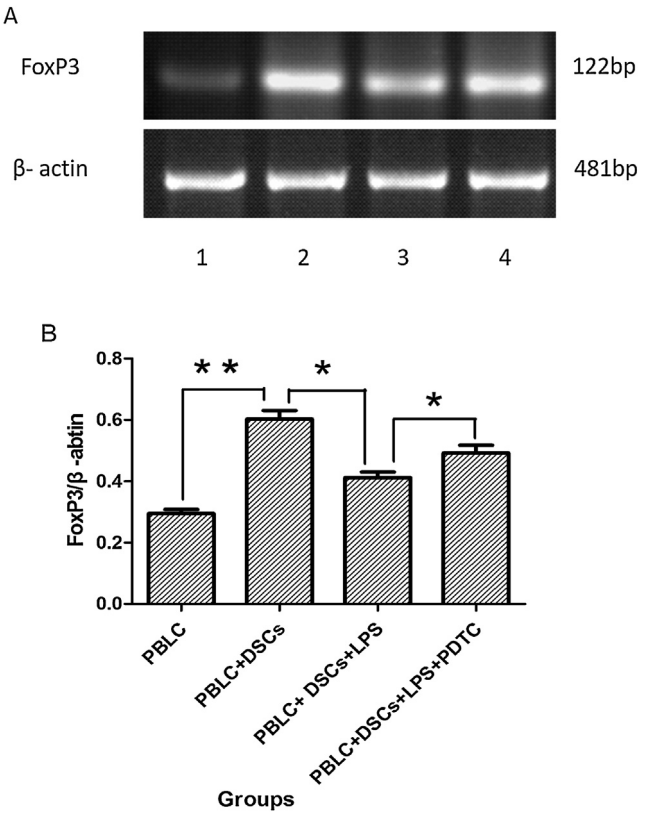
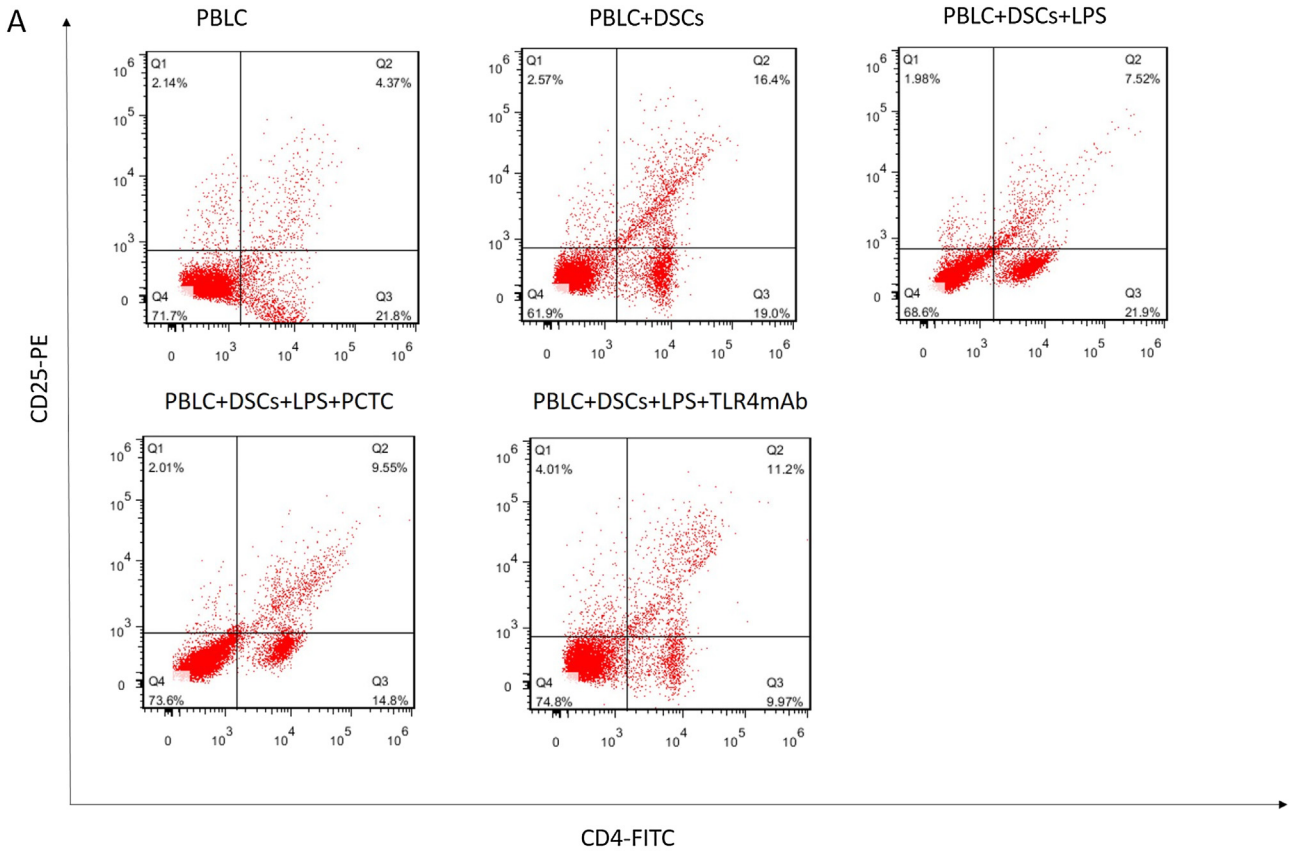


Fig. 4. Expression of FoxP3 mRNA in different cultivation groups. 1. Control group; 2. DSCs + PBLC; 3. DSCs + PBLC + LPS; 4. DSCs + PBLC + LPS + PDTC.

diseases and maternal immune tolerance [16]. In normal pregnancy, the peripheral blood Treg cells increased from the beginning of early pregnancy and they migrate to the maternal-fetal interface under the effects of HCG secreted by trophoblast cells and a variety of chemokines [17–19].Treg cells in the maternal-fetal interface are the primary cells that regulate maternal-fetal immune balance, and studies found that recurrent miscarriage, eclampsia and other patients existed reduction of decidual Treg cells [20,21]. In addition, there is a decrease number or function of Treg cells in a variety of autoimmune diseases and inflammatory diseases [22]. Therefore, intervention of Treg cells may be a target for the treatment of a variety of autoimmune diseases and inflammatory diseases.

In normal pregnancy, endometrium in the secretory phase generated decidualization under the combined effects of estrogen and progesterone along with the formation of DSCs. DSCs have a variety of biological functions, they are not only the main component of decidual, but also the interface of mother and the fetus to produce immune response [23]. DSCs are function as regulators for T cells entering the maternal-fetal interface [24]. The study of DSCs is a hotspot in reproductive immunology research. Tom Erkers et al. [25] found that DSCs inhibited the allogeneic lymphocyte response mainly in a contact-dependent manner and promoted cell proliferation of Treg cells. In this study, we used co-cultivation of DSCs and peripheral lymphocytes to simulate the microcirculation of maternal-fetal interface, and then to detect the expression of CD4 and CD25 on Treg cells which served as a specific marker for Treg cells [26]. It was found that DSCs could promote the differentiation of PBLC into Treg cells, simultaneously, we found that the expression of FoxP3 was significantly increased, suggesting that DSCs could promote the differentiation of Treg cells. The mechanisms may be related to increased proportion of Treg cells induced by promoting FoxP3 expression.



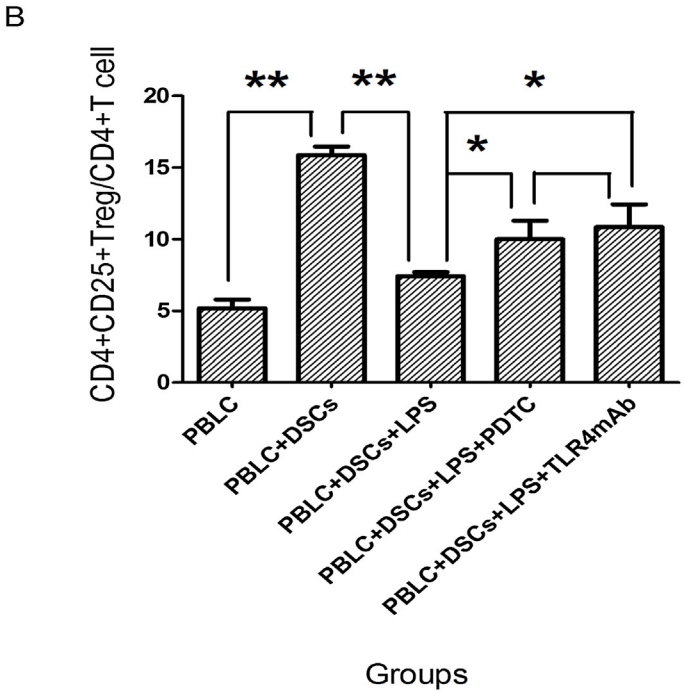


Fig. 5. Proportion of $CD4^+CD25^+$Treg/$CD4^+$T cells in each group.

TLR4 is a member of the Toll-like receptor family. LPS is a cell wall component of pathogenic microorganisms that specifically activates TLR4 and activates the downstream NF-κB signaling pathway through the MyD88-dependent and MyD88-independent pathway to produce IL-6, TNF-α and other cytokines [27]. Previous studies have found that TLR4 expressed in immune cells, however, recent study found that the trophoblast cells and decidual cells in the maternal-fetal interface also expressed TLR4. Moreover, TLR4 expressed higher in decidual cells than in trophoblast cells. When maternal-fetal interface suffered from infection or inflammation and other stimulation, immune tolerance imbalance led to the occurrence of pregnancy complications [28]. After stimulating DSCs with

Table 2
Proportion of $CD4^+CD25^+$Treg/$CD4^+$T cells in each group (%, $x(\_)\pm s$, $n=4$).

Groups	$CD4^+CD25^+$Treg/$CD4^+$T
PBLC cultivation group	5.200 ± 0.593
Co-cultivation of DSCs + PBLC	$15.875 \pm 0.602^*$
Co-cultivation of DSCs + PBLC + LPS	$7.418 \pm 0.291^{*\#}$
Co-cultivation of DSCs + PBLC + LPS + PDTC	$9.953 \pm 1.222^{*\#\triangle}$
Co-cultivation of DSCs + PBLC + LPS + TLR4mAb	$10.860 \pm 1.582^{*\#\triangle}$

Compared with the control group, $^*P < 0.05$; compared with the co-cultivation of DSCs + PBLC group, $\#P < 0.05$; compared with the co-cultivation of DSCs + PBLC + LPS group, $\triangle P < 0.05$.

different concentrations of LPS to simulate the microenvironment of inflammatory state, we found that the expression of TLR4 was significantly up-regulated with the raised concentration of LPS. It has been found that TLR4 signaling pathway can directly or indirectly affect the proliferation and function of Treg cells. Zhang N [29] found that activation of TLR4 after allogeneic islet transplantation inhibited the expression of FoxP3 and promoted the secretion of IL-6, thus inhibited the production of $FoxP3^+$ Treg cells. Opitz et al. found that TLR4 attenuated the immunosuppressive function of bone marrow stromal cells and reduced the production of Treg cells [30]. However, the effect of TLR4 on Treg cells in maternal-fetal interface is unclear. It was reported that 100 ng/ml of LPS had no irreversible effects on the activity of DSCs [14]. We used 100 ng/ml of LPS to stimulate DSCs and found that the expression of FoxP3 mRNA was significantly decreased as well as the number of Treg cells compared with the co-cultivation group. But the proportion of Treg cells subgroup was significantly increased after TLR4 neutralized antibody blocking TLR4 signal pathway, suggesting that DSCs could play a regulatory role in Treg cells through the TLR4 signaling pathway. Schuster M et al. [31] found that NF-κB atypical inhibitor IκB (NS) could bind with FoxP3 gene promoter to facilitate FoxP3 expression, resulting in increased Treg cells. Our study found that after treating DSCs with LPS, IκBα protein and FoxP3 mRNA expression was significantly reduced as well as the proportion of Treg cells decreased significantly, suggesting that TLR4 can inhibit transcription of FoxP3 and reduce differentiation of Treg cells. Furthermore, we used a specific inhibitor of NF-κB, PDTC to block NF-κB so that inhibiting the ubiquitination of IκB-α, we found that the expression of IκB-α protein and FoxP3 was significantly increased and the proportion of Treg cells raised evidently. After blocking the NF-κB signaling pathway with PDTC, the number of Treg cells increased but still less than that of the co-cultivation group, indicating that NF-κB signaling pathway was not the only way through which TLR4 affected Treg cells.

Our results suggest that DSCs can increase the proportion of Treg cells in peripheral blood of reproductive women. After treated DSCs with LPS, the expression of TLR4 increased and the differentiation of Treg cells induced by DSCs was inhibited, suggesting that DSCs plays an immunoregulatory role in maternal-fetal interface. The effect may proceed via promoting NF-κB activation and inhibiting transcription of FoxP3. DSCs, Treg and TLR4 are of great significance in pregnancy tolerance. We have explored their relationship, whereas their interaction and the underlying mechanisms still call for more research.

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