Journal of Reproduction and Development, Vol. 67, No 6, 2021

Original Article

Intrauterine administration of peripheral blood mononuclear cells (PBMCs) improves embryo implantation in mice by regulating local Treg/Th17 cell balance

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Abstract. Immune imbalance of Treg/Th17 cells may contribute to recurrent implantation failure (RIF) during *in vitro* fertilization and embryo transfer (IVF-ET). In this study, we sought to determine the effect of intrauterine administration of mouse PBMCs prior to embryo implantation on endometrial receptivity and embryo implantation, and examine the underlying mechanism of Treg/Th17 cell balance following intrauterine administration of PBMCs. Pregnant mice were randomly divided into three groups: control group, embryo implantation dysfunction (EID) group, and EID with PBMCs group, and the number of embryo implantation sites was recorded during early pregnancy (Pd7.5). The balance of Treg/Th17 cells in the peripheral blood, spleen, and local implantation sites was detected during the peri-implantation period (Pd4.0) and early pregnancy (Pd7.5). The EID group demonstrated a significant decrease in the number of embryo implantation sites, while the EID with PBMCs group demonstrated higher number of embryo implantation sites compared to the EID group. The balance of Treg/Th17 cells in the peripheral blood and spleen tissues was not significantly different between the aforementioned groups. However, the local uterine ratio of the Treg/Th17 cells increased in the EID with PBMCs group compared to that in the EID group. Collectively, we found that intrauterine administration of PBMCs prior to embryo implantation effectively promotes embryo implantation rates. This may be attributed to the improvement in the local immune balance of Treg and Th17 cells compared with the overall immune balance.

Key words: Peripheral blood mononuclear cells, Recurrent implantation failure, Th17, Treg

(**J. Reprod. Dev. 67:** 359–368, 2021)

In vitro fertilization (IVF) techniques have advanced markedly over the past 40 years since the birth of Louise Brown, the first baby to be conceived by IVF, in 1978. Although IVF is a reliable choice for unresolved infertility, several disadvantages have emerged over the years in its application [1]. Recurrent implantation failure (RIF) following embryo transfer (ET) is a serious obstacle that limits the success rate of IVF. Despite the lack of a standardized definition, RIF is currently defined as unsuccessful implantation or failure of clinical pregnancy after two consecutive transfer cycles of at least four cleavage stage embryos or two high-grade blastocysts with an incidence of approximately 10% [2]. In view of the challenges encountered with the incidence of RIF, additional efforts are urgently required to increase successful fertilization and implantation.

The etiology of RIF is complex and the causative factors include gamete/embryo factors, chromosomal abnormalities, unrecognized uterine pathology, immune factors, inadequate culture conditions, and improper ET methods [2, 3]. Embryo implantation is a complex and intricate process that is regulated by several factors. A balance between 'immune rejection' and 'immune tolerance' at the local implantation site is indispensable for pregnancy maintenance [4]. Accumulating evidence has demonstrated that the balance of immune cells, including T-regulatory cells (Tregs), T-helper cells (Th1/2,Th17), uterine natural killer (uNK) cells, uterine mast cells (uMCs), macrophages, and dendritic cells at local implantation site contributes to successful embryo implantation [5–9].

The treatment of RIF is largely empirical and based on poor scientific evidence. In the past few decades, several studies have focused on immunological therapy for RIF. Intradermal lymphocyte immunotherapy (LIT) has been suggested as an suitable approach to prevent RIF [10]. However, within a few years following LIT, certain adverse effects have been reported, such as transfusion-associated disorders, autoimmune disorders, and gestational pathology [11]. In 2006, Yoshioka *et al.* reported for the first time that intrauterine administration of peripheral blood mononuclear cells (PBMCs) improves the implantation rate, clinical pregnancy rate, and birth rate in women with RIF [12]. Since then, a limited number of similar studies have shown the promising benefits of this method with the combined use of PBMCs [13, 14].

PBMCs mainly consist of tlymphocytes, blymphocytes, natural killer cells, monocytes, and dendritic cells. Activated CD4+ T cells are further divided into Th1, Th2, Th9, Th17, and Th22, follicular helper cells (Tfh), and regulatory T cell (Treg) subsets. This classification is based on the production of specific cytokines, as well as on the expression of specific transcription factors and surface markers [15]. Various mechanisms have been proposed to explain the implantation-promoting effects of autologous PBMCs. PBMC treatment was reported to induce the production of several cytokines, such as IL-1α, IL-1β, and TNF-α, which have a positive impact on endometrial receptivity and affect blastocyst attachment and invasion [16]. In addition, PBMC treatment may modulate the local immune system to maintain the balance between the pro-inflammatory

Received: January 13, 2021
Accepted: September 10, 2021
Advanced Epub: October 7, 2021
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response and maternal immunologic tolerance [17]. Among the various classes of immune cells, CD4+ T cells are important players in the establishment of pregnancy. In particular, the balance between Th1 and Th2 cells is the most widely studied function of T cells. Th1/ Th2 cell imbalance has been predicted in patients with unexplained RIF [6]. Furthermore, the Th1/Th2 cell paradigm has been applied to Treg/ Th17 cells [18]. It is well-known that the imbalance of circulating Treg and Th17 cells and the consequent changes in the expression levels of the associated cytokines may be implicated in the pathogenesis of RIF and in recurrent spontaneous abortion (RSA) [19, 20]. Our previous study confirmed that intrauterine administration of autologous PBMCs *in vitro* improved the embryo implantation rate selectively in patients with RIF [21].

Mifepristone, a classical antagonist of progestin, destroys endometrial receptivity and inhibit blastocyst implantation. It is used to induce abortion or as an effective emergency contraceptive [22, 23]. Additionally, it has often been used to induce embryo implantation failure or polycystic ovary syndrome in experimental animal models [24–26]. The expression of progesterone receptor genes and proteins in the endometrium was ubiquitously decreased following mifepristone administration [23, 27]. The well-known endometrial receptivity markers, such as leukemia inhibitory factor (LIF) and integrin αvβ3, are regulated by progesterone directly or indirectly [28]. The effect of mifepristone on the local immune microenvironment of the endometrium has been reported occasionally. Chen *et al*. demonstrated that mifepristone directly augmented the cytotoxicity of human uterine natural killer (uNK) cells [29]. Gao *et al*. found that the uNK cell subset in the rat endometrium was reduced after mifepristone treatment [30]. Jaya *et al*. reported that the level of uterine monocyte chemotactic protein-3 (MCP3), a pro-inflammatory and Th1 effector, was increased after the administration of mifepristone [31]. Additionally, Li Y *et al*. suggested that mifepristone regulates DC function by decreasing TGF-β expression *in vitro*, which further results in the downregulation of Foxp3 and IL-10 in Tregs [32]. Notably, progesterone suppresses IL-17 expression [33, 34]. We speculated that when the progesterone receptor is antagonized, IL-17 production may be increased. Thus, mifepristone affects the uterine immune environment. However, the process is complex and requires further study.

In our previous study, we successfully established an embryo implantation dysfunction (EID) model in mice using small doses of mifepristone. We further demonstrated that intrauterine administration of mouse PBMCs prior to embryonic implantation significantly increased the embryo implantation rate [35]. However, the specific mechanism and whether PBMCs participate in the regulation of immunity remain unclear. We hypothesized that intrauterine administration of PBMCs may influence the balance between Tregs and Th17 cells at the local implantation site. Consequently, the objective of this study was to identify the potential immunological mechanism of implantation-promoting effects following the intrauterine administration of PBMCs.

Materials and Methods

Experimental animals

SPF grade mature Kunming mice (6–8 weeks old, weighing 24–28 g) were purchased from the Animal Center of the Tongji Hospital, Wuhan, China. The female and male animals were maintained separately for a week in an environment-controlled room (12 h light/12 h dark photoperiod, 18–22°C, 70–85% relative humidity) with free access to food and water. These conditions were used to acclimatize the mice. Subsequently, estrogen-producing females were caged with males (ratio 2:1) overnight and checked for vaginal plugs the following morning. Female animals with vaginal plugs were considered to be on day 0.5 of pregnancy (Pd0.5). The study protocol was approved by the Institutional Animal Care and Use Committee of the Tongji Medical College at the Huazhong University of Science and Technology. The experiments were performed in accordance with the appropriate guidelines for animal handling.

Groups and treatments

Female mice were randomly divided on Pd0.5 into three groups: control group, EID group, and EID with PBMCs group. On Pd1.5, PBMCs were isolated from total blood of non-pregnant female mice (see below for detailed steps) and washed with phosphate buffered saline (PBS) buffer. The washed PBMCs were resuspended in basal culture medium without fetal bovine serum (FBS) or fetal calf serum (FCS). Then the PBMCs (1–2 × 10^6 in 2.5 µl) were gently injected surgically into the bilateral uterine horn of mice in the EID with PBMCs group using a microsyringe. The mice in the other groups received the same amount of basal culture medium (Roswell Park Memorial Institute 1640 (RPMI 1640; C11875500BT, Gibco®, Waltham, MA, USA). The EID and the EID with PBMCs groups were obtained by injecting the mice with 0.08 mg mifepristone (H10950003, Zizhu Pharmaceutical, Beijing, China) subcutaneously at 0900 h on Pd3.5.

Sample collection

On Pd4.0 (the peri-implantation period), half of the pregnant mice in each group were sacrificed at 2100 h. to evaluate the immune environment. The remaining mice were sacrificed at 0900 h on Pd7.5 (embryo formation period) to compare the number of blastocysts. The following samples were collected: (i) peripheral blood (to isolate mononuclear cells for flow cytometric analysis); (ii) spleen (single-cell suspension cells for flow cytometric analysis); (iii) uterus (the embryos were excluded and the cells were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and prepared into 4-µm-thick paraffin sections for immunofluorescence staining). The sections were cryopreserved at –76°C for real-time PCR and western blot analyses.

Isolation of PBMCs

Blood was drawn from the eyeballs of non-pregnant mice and transferred to anticoagulant tubes. PBMCs were isolated by density centrifugation using a Mouse Peripheral Blood Lymphocyte Isolation Kit (LTS1092; Tianjinhaoyang Biological Manufacture Co., Ltd., Tianjin, China) according to the manufacturer's instructions. The second layer of milky PBMCs was collected according to the manufacturer's instructions (Pharm Lyse™ lysing buffer; BD Biosciences, San Diego, CA, USA). Subsequently, the cells were washed twice with PBS. Finally, the PBMCs (1 × 10^6 cells/ml) were suspended in RPMI 1640 (Gibco®) supplemented with 10% FBS (10270160; Gibco®), 50 U/ml penicillin, and 50 µg/ml streptomycin (15140122; Invitrogen, Waltham, MA, USA).

Flow cytometry for Treg and Th17 cells

Fresh peripheral blood and spleen samples were obtained from pregnant mice on Pd4.0 and Pd7.5. T cells were isolated from the peripheral blood and spleen from leukocyte and single-cell suspension, respectively. The spleen was placed in a disposable sterile petri dish containing 1–2 ml PBS and crushed gently until the tissue pieces turned white in color. Subsequently, 3 ml of PBS was added to the

petri dish. The cell suspension (4–5 ml) was transferred into a new petri dish following filtration through a sterile fine-wire mesh (74 μm). Subsequently, the cell suspension was collected and centrifuged at 1100 rpm for 5 min. The peripheral blood and spleen cell suspensions were incubated with lysis buffer (420301; BD Bioscience) for erythrocyte lysis according to the manufacturer's protocol. Finally, the cells were centrifuged, resuspended, and counted (1×10^6 cells/ml) for flow cytometric analysis.

To increase the sensitivity of detection of IL-17A by intracellular staining, a leukocyte activation cocktail was added (423303; BD Bioscience; 2 μl/1×10^6 cells/ml cell culture) to the 24-well cell culture plates. The plates were incubated under regular culture conditions (37°C, 5% CO_2, high humidity) for 6–8 h. Subsequently, the cells were surface-labeled with anti-mouse CD3-PerCP-Cy5.5 (100205; BD Bioscience), anti-mouse CD4-FITC (116003; BD Bioscience), and anti-mouseanti-Mouse CD25-APC (102011; BD Bioscience) for 30 min at 4°C in the dark. The cells were subsequently washed, fixed and permeabilized by the CytoFix/CytoPerm Solution Kit or the BD Pharmingen™ Transcription Factor Buffer Set. The permeabilized cells were labeled by anti-Mouse IL-17A-Alexa 647 (506915; BD Bioscience) and anti-mouse Foxp3-PE (126404; BD Bioscience) for 35–45 min at 4°C in the dark. Finally, the treated cells were washed and resuspended in PBS-FBS solution (200 μl) for further analysis. The samples were read on a FACSCanto II flow cytometer (BD Bioscience) and analyzed using the FACSDiva™ software.

Immunofluorescence staining

Paraffin sections of the uterine tissues were boiled in citrate buffer (10 mM sodium citrate, 10 mM citric acid, pH 6.0) in a microwave oven at 92–98°C for 15 min for antigen retrieval. The slides were then blocked with 5% BSA for 30 min at room temperature (25°C). Subsequently, the tissues were incubated with the following primary antibodies: anti-Foxp3 monoclonal antibody (Abcam, Cambridge, MA, ab20034; 1:200) and anti-RORγt monoclonal antibody (Abcam, ab207082, 1:200) overnight at 4°C. The tissues were then incubated with fluorescein-conjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies for 1 h at 4°C in the dark. Subsequently, the nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, G1012; Xavier Biotechnology Co., Ltd., China) for 10 min. The slides were washed with PBS and mounted with an anti-fluorescence quenching agent. Optical microscopy (Olympus IX73; Tokyo, Japan) was used to capture images.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the frozen uterine tissues using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration was measured by spectrophotometry, and equal amounts (2 μg) of RNA were used for cDNA synthesis using a reverse transcription kit (FSQ-101; Toyobo, Japan). A total of 2 μl of cDNA was amplified with SYBR® Green Universal PCR Master mix (QPK-201; Toyobo, Japan) and specific primers for each gene. The primer sequences used for the detection of *Foxp3*, *RORγt*, *IL-10*, *IL-17*, *IL-21*, *IL-22*, and *GAPDH* mRNAs are as follows:

Foxp3 F:5'-GGCAGAGAGGTATTGAGGGTG-3', R:5'CTTTCTT CTGTCTGGAG TGGC-3'; *RORγt* F: 5'-TACCCTACTGAGGAGGACAGG-3', R: 5'-AACCCCGTAGTGGA TCCCAG-3'; *IL-10* F: 5'- GGCCCAGAAATCAAGGAGCA-3', R: 5'-AATCG ATGACAGCGCCTCAG-3'; *IL-17* F: 5'-CACCGCAATGAAGACCCTGA-3', R: 5'-TT CCCTCCGCATTG ACACAG-3'; *IL-21* F: 5'- GGCTCTCGTTCCCACAGATG-3', R: 5'-CGTCTATAGTGTCCGGCGTC-3'; *IL-22* F: 5'-TGTGCGATCTCTGATGGCTG-3', R: 5'-GTCACCGCTGATGTGACAGG-3'; *GAPDH* F: 5'- TGCACCACCAACTGCTT AGC-3', R: 5'-GGCATGGACTGTGGTCATGAG-3'. qRT-PCR was performed in an Mx 3000p™ real-time PCR System (Applied Biosystems Inc., Foster City, USA) according to the following protocol: 95°C for 5 min, 39 cycles at 60ºC for 30 sec, and 72°C for 30 sec, followed by 95°C for 15 sec. All reactions were performed in triplicate. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt represents the difference between the calibrated Ct values of the test and control samples. The mRNA expression in each sample was normalized to that of *GAPDH* mRNA.

Western blotting

Total protein was extracted from the frozen uterine tissues using RIPA lysis buffer (G2002; Xavier Biotechnology Co., Ltd., China) containing a protease inhibitor cocktail (G2006; Xavier Biotechnology Co., Ltd.). Protein concentration was determined using the BCA Protein Assay Kit (G2026; Xavier Biotechnology Co., Ltd.) following the manufacturer's instructions. The 5 × sodium dodecyl sulfate (SDS) loading buffer was added to the samples, which were then boiled for 12 min at 95°C and cryopreserved at −80°C until further use. Initially, 50 μg of the protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS–PAGE, G2003; Xavier Biotechnology Co., Ltd.) and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated in TBST (50 mM Tris–HCl, 150 mM NaCl, and 0.1% [v/v] Tween-20 [G5058; Xavier Biotechnology Co., Ltd., China]) containing 5% skimmed milk at room temperature (25°C) for 1 h and subsequently incubated with anti-Foxp3 antibody (Abcam, ab215206; 1:1,000), anti-RORγt polyclonal antibody (Biorbyt, Cambridge, Britain, orb385620; 1:1,000), or anti-GAPDH antibody (Abcam, ab37168; 1:7,000) overnight at 4°C. The following day, the membranes were rinsed in TBST and incubated with HRP-conjugated goat anti-rabbit antibody (Aspen, Johannesburg, South Africa, AS1107; 1:10,000) at room temperature (25°C) for 1 h. Finally, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL, G2014; Xavier Biotechnology Co., Ltd.) according to the manufacturer's protocol and exposed to X-ray films (Kodak, Rochester, New York, USA). Densitometry analysis was performed using ImageLab software (Bio-Rad Laboratories, Inc., Berkeley, California, USA).

Statistical analysis

The data are expressed as mean ± standard deviation (SD). The differences between multiple groups were analyzed using the paired *t*-test and one-way analysis of variance. The number of pregnant mice was analyzed using the chi-square test. In all cases, a P value lower than 0.05 ($P < 0.05$) was considered significant and a P value lower than 0.01 ($P < 0.01$) was considered highly significant. Each experiment was repeated three times.

Results

Intrauterine administration of PBMCs improves the embryo implantation rate in mice with embryonic implantation dysfunction

To examine the effect of intrauterine administration of PBMCs on embryo implantation, the average blastocyst number in pregnant mice in each group was compared on Pd7.5 (Fig. 1). The number of

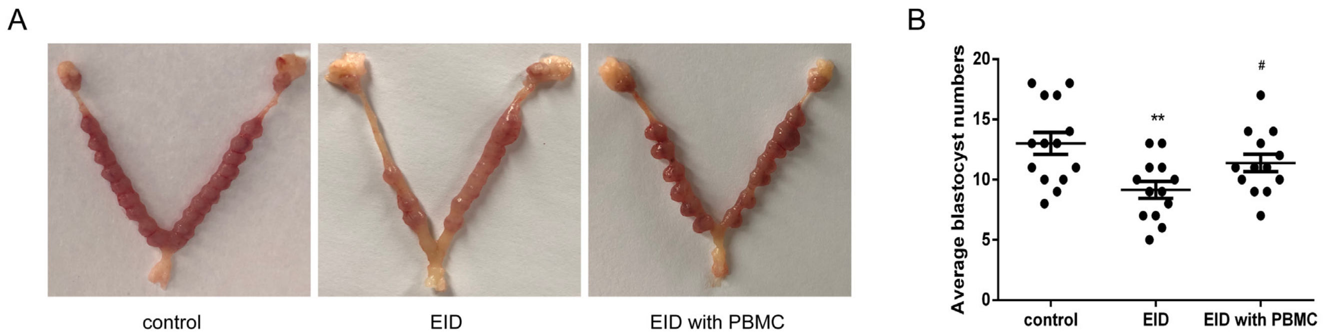

Fig. 1. The three groups of embryo implantation. (A) Number of blastocysts implanted in each group on Pd7.5. Control group, 19 implanted blastocysts; EID group, 10 implanted blastocysts; EID with PBMCs group, 13 implanted blastocysts. (B) Statistical analysis of the average number of blastocysts in control (n = 14), EID (n = 13) and EID with PBMCs (n = 13) groups. The data are presented as the mean ± standard error of mean (SEM). ** $P < 0.01$ *vs*. control group, # $P < 0.05$ *vs*. EID group.

mice with effective pregnancy in each group was as follows: control group (n = 14), EID group (n = 13), and EID with PBMCs group (n = 13). Notably, the average blastocyst number in the EID group was significantly decreased compared to that in the control group (13.00 ± 0.91 *vs*. 9.15 ± 0.70, $P < 0.01$). Following intrauterine administration of PBMCs, the average blastocyst number was increased compared to that in the EID group (9.15 ± 0.70 *vs*. 11.38 ± 0.73, $P < 0.05$). In addition, the pregnancy rates of the pups in the control, EID, and EID with PBMCs groups were 0.867, 0.870, and 0.879, respectively, with no statistical difference.

Intrauterine administration of PBMCs does not affect the differentiation of Treg and Th17 cells in spleen tissues and peripheral blood

To explore the effect of intrauterine administration of PBMCs on Treg and Th17 cell differentiation in the spleen and peripheral blood, we analyzed the proportion and ratio of Treg and Th17 cells by flow cytometry. On Pd4.0 and Pd7.5, 12 mice were tested in each group. The gating strategies for Treg and Th17 cells are shown in Fig. 2A. Them gating and percentage of Treg and Th17 cells in each group are shown in Fig. 2B. The percentage of each subpopulation was determined by the proportion of $CD4^+$ T cells (Fig. 3).

In the spleen, the results showed that the percentages of Treg and Th17 cells were not m different among the three groups on both Pd4.0 and Pd7.5. Moreover, the ratios of Treg and Th17 cells were analyzed in each group. On Pd4.0 and Pd7.5, the ratios exhibited no significant differences among the three groups. In the peripheral blood, there was no significant difference in the percentage of Treg cells among the three groups on both Pd4.0 and Pd7.5. The percentage of Th17 cells was higher in the EID group than in the control group (5.54 ± 0.64% *vs*. 4.03 ± 0.32%, $P < 0.05$). However, the difference in Th17 cells in the EID with PBMCs group was not statistically significant compared to that in the EID group. In addition, the ratio of Treg and Th17 cells was not significantly different between Pd4.0 and Pd7.5.

Intrauterine administration of PBMCs modulates Treg and Th17 cells associated transcription factors and cytokine expression in the uterus

To assess the effect of intrauterine administration of PBMCs on the uterine immune microenvironment, we evaluated the expression of Treg and Th17 cell associated nuclear transcription factors (Foxp3 and RORγt). Twelve mice in each group were tested on Pd4.0 and Pd7.5. Immunofluorescence staining revealed that the expression of Foxp3 was reduced in the EID group compared to that in the control group on Pd4.0, whereas the expression of RORγt was increased. Interestingly, these changes were partially reversed in the EID with PBMCs group (Fig. 4). Western blotting (Fig. 5A–C) and RT-PCR (Fig. 5D–E) revealed significant differences in the protein and mRNA, levels of Foxp3 and RORγt, respectively, on Pd4.0. On Pd7.5, the results of the immunofluorescence staining revealed a small change in the expression of Foxp3 and RORγt, which was inconsistent with the results of western blotting and RT-PCR assays. We further found that both protein and mRNA levels of Foxp3 and RORγt were altered with a trend similar to that noted on Pd4.0. However, except for the *Foxp3* mRNA levels in the control and EID groups, the aforementioned changes on Pd7.5 exhibited no significant differences.

Furthermore, the mRNA levels of related cytokines, which play crucial roles in the regulation of the number and function of Treg and Th17 cells, were measured (Fig. 5F–I). On Pd4.0, *IL-10* mRNA levels were significantly lower in the EID group than in the control group ($P < 0.05$). It increased significantly in the EID with PBMCs group compared to that in the EID group ($P < 0.05$). On Pd4.0, *IL-17* and *IL-22* mRNA levels were significantly higher in the EID group than in the control group ($P < 0.05$). It decreased significantly in the EID with PBMCs group compared to that in the EID group ($P < 0.05$). *IL-21* mRNA level was decreased in the EID group and partially reversed in the EID with PBMCs group on Pd4.0. However, these differences were not significant. The aforementioned differences observed on Pd4.0 were not present on Pd7.5.

Discussion

The present study was a randomized and controlled comparative study that focused on the effects and potential mechanism of intrauterine administration of PBMCs on embryo implantation. We successfully constructed an EID model, in which glandular secretory differentiation was delayed and decidualization was impaired by mifepristone. The embryo implantation rate in the EID group was lower than that in the control group. Following intrauterine administration of PBMCs prior to embryonic implantation, the embryo implantation rate was significantly increased, which was similar to the results obtained in our previous study [35]. This is consistent with the findings of previous studies demonstrating that intrauterine administration of PBMCs improved the pregnancy rate of human subjects undergoing ET therapy [12–14]. Based on these findings, we aimed to explore

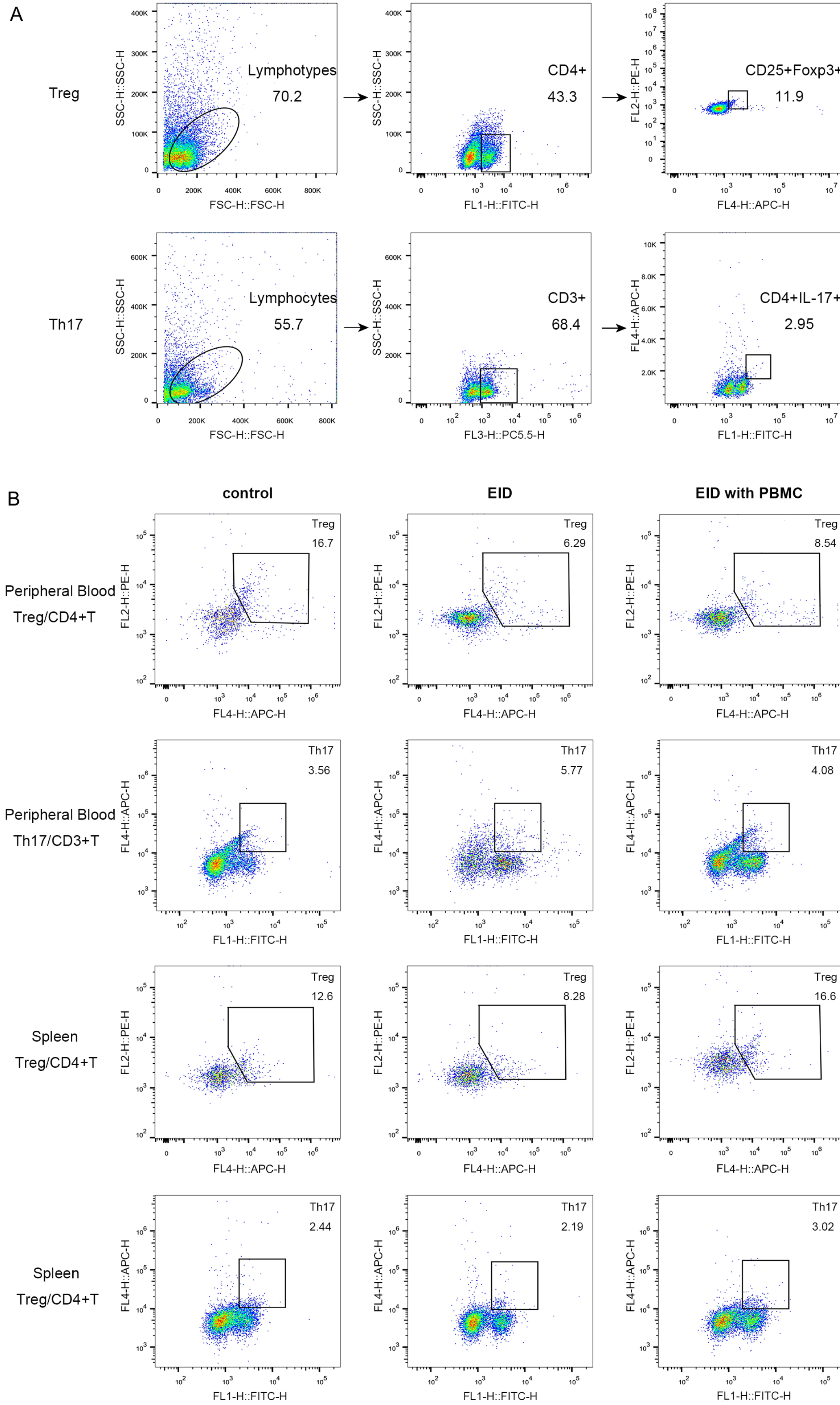

Fig. 2. Determination of peripheral Treg and Th17 cells by flow cytometry on Pd4.0 and Pd7.5. (A) Gating strategies for Treg cells and Th17 cells. For Treg quantification, CD4+ cells were first selected in lymphocyte population followed by gating of CD25+ FOXP3+ cells within CD4+ lymphocytes. For Th17 quantification, CD3+ cells were first selected in lymphocyte population followed by gating of CD4+ IL-17+ cells within CD3+ lymphocytes. (B) The respective gating and percentage of Treg and Th17 cells in the spleen tissues and peripheral blood samples of the different groups.

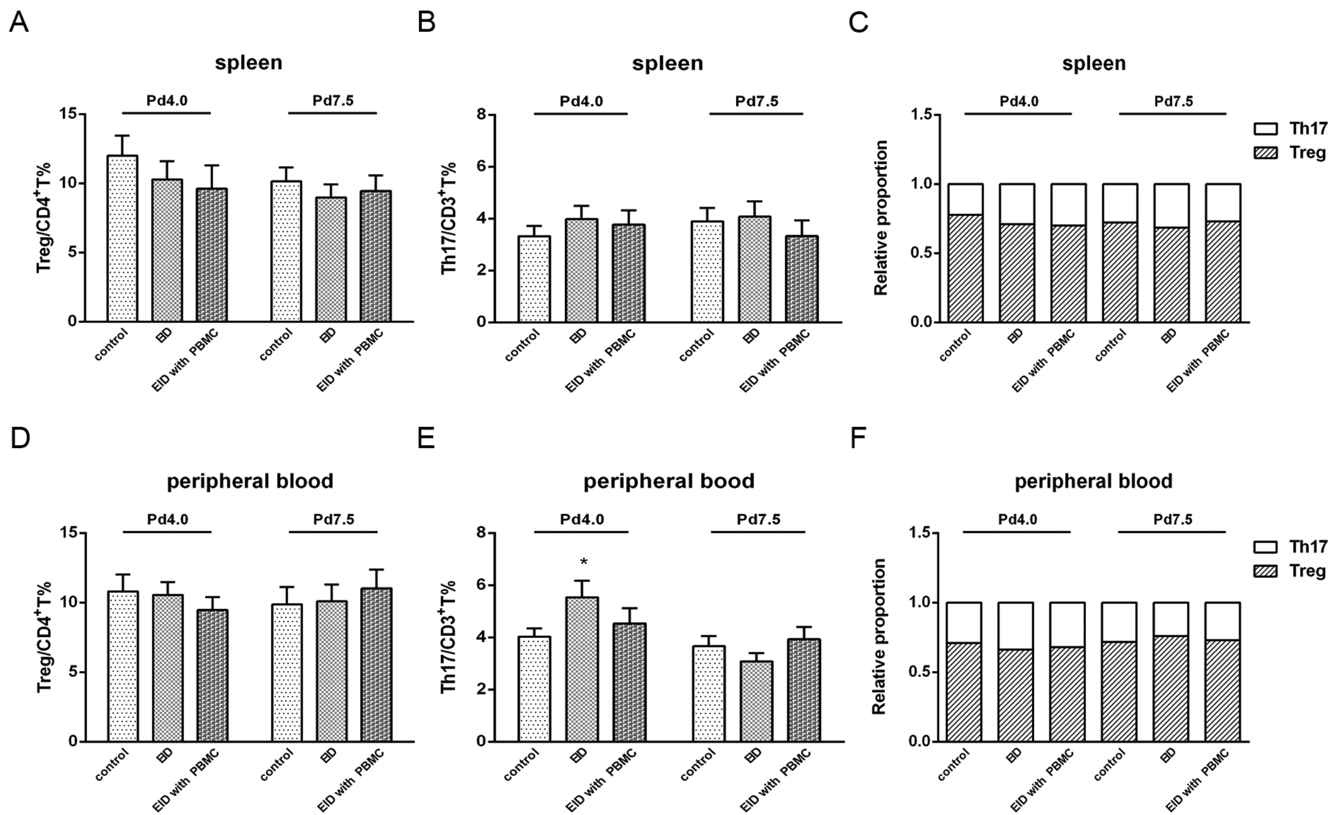

Fig. 3. Flow cytometric analysis. On Pd4.0 and Pd7.5, ten mice were tested in each group. The average percentage of Treg cells was determined in the spleen tissues (A), and peripheral blood (D). The average percentage of Th17 cells was determined in the spleen tissues (B), and peripheral blood (E). Relative proportions of Treg and Th17 cells in the aforementioned biological matrices (C, F). The data are presented as the mean ± SEM. * P < 0.05 *vs*. control group.

the underlying mechanism of the implantation-promoting effects of intrauterine administration of PBMCs.

Optimal implantation is a prerequisite for successful pregnancy. An adequate decidual response is critical to the success of implantation, ensuring embryo acceptance with a well-tolerated immune microenvironment. Initially, the semi-allogeneic fetus produces an immune response to the mother's immune system. Maternal immune cells reject alloantigens and are actively involved in several processes of establishing, sustaining, and terminating pregnancy [36]. Inflammation-like processes are crucial for tissue growth, remodeling, and differentiation of the decidua. As conception proceeds, the inflammatory response must be controlled and reversed to an anti-inflammatory condition for implantation to progress [5]. Among the numerous types of immune cells, Th17 cells, which represent a vital lineage of pro-inflammatory T-helper cells, have been acknowledged as a barrier to the induction of immune tolerance in pregnancy [37]. They are characterized as cells that preferentially produce interleukins, including IL-17A, IL-17F, IL-21, and IL-22. RORγt, the steroid receptor–type nuclear receptor, induces the development of Th17 cells and production of IL-17, which are commonly used to identify Th17 cells, along with specific cytokines (IL-17 and IL-21) [38–41]. Treg cells are known to inhibit immune responses by producing the anti-inflammatory cytokines, IL-10 and TGF-β. They are generated from naïve CD4+ T cells following antigen stimulation with TGF-β and IL-2. This process activates the expression of the specific transcription factor Foxp3 [42]. Previous clinical research has shown that increasing number of Th17 cells and decreasing number of Treg cells in circulation may contribute to RIF and RSA [19, 43, 44]. These findings have also been established in mouse models with RIF and RSA [45, 46]. However, Sadeghpour *et al*. found that when local Th17 cells decreased and Treg cells increased, the pregnancy rate in patients with RIF did not improve significantly [41]. This is not entirely consistent with the findings of Ahmadi *et al*. [44, 47]. Therefore, the precise underlying mechanism needs to be studied further.

Based on the data from the research studies mentioned above, we examined the peripheral and local balance of Treg/Th17 cells among the control, EID, and EID with PBMC groups. During the preimplantation period (Pd4.0), the ratio of Treg and Th17 cells was assessed in peripheral blood and spleen tissues by flow cytometry. The expression levels of typical cytokines and transcription factors in the local uterine tissues were identified by immunofluorescence, RT-PCR, and western blot assays. These tests were similarly carried out on Pd7.5. On Pd4.0, the EID with PBMCs group exhibited significantly higher production of Treg-type cytokine (IL-10) and transcription factor (Foxp3) at sites of embryonic implantation compared to the EID group. The opposite results were observed in Th17 cells that produced the cytokines IL-17 and IL-22, and the transcription factor RORγt. However, the expression level of IL-21 was not consistent with that of IL-17 and IL-22. Possible reasons include the following: 1) Other immune cells that produce IL-21 may have an effect; 2) Inhibition of the effect of progesterone did not affect the production of IL-21. A previous study also reported inconsistent results with regard to changes in levels of the two cytokines [48]. Notably, the

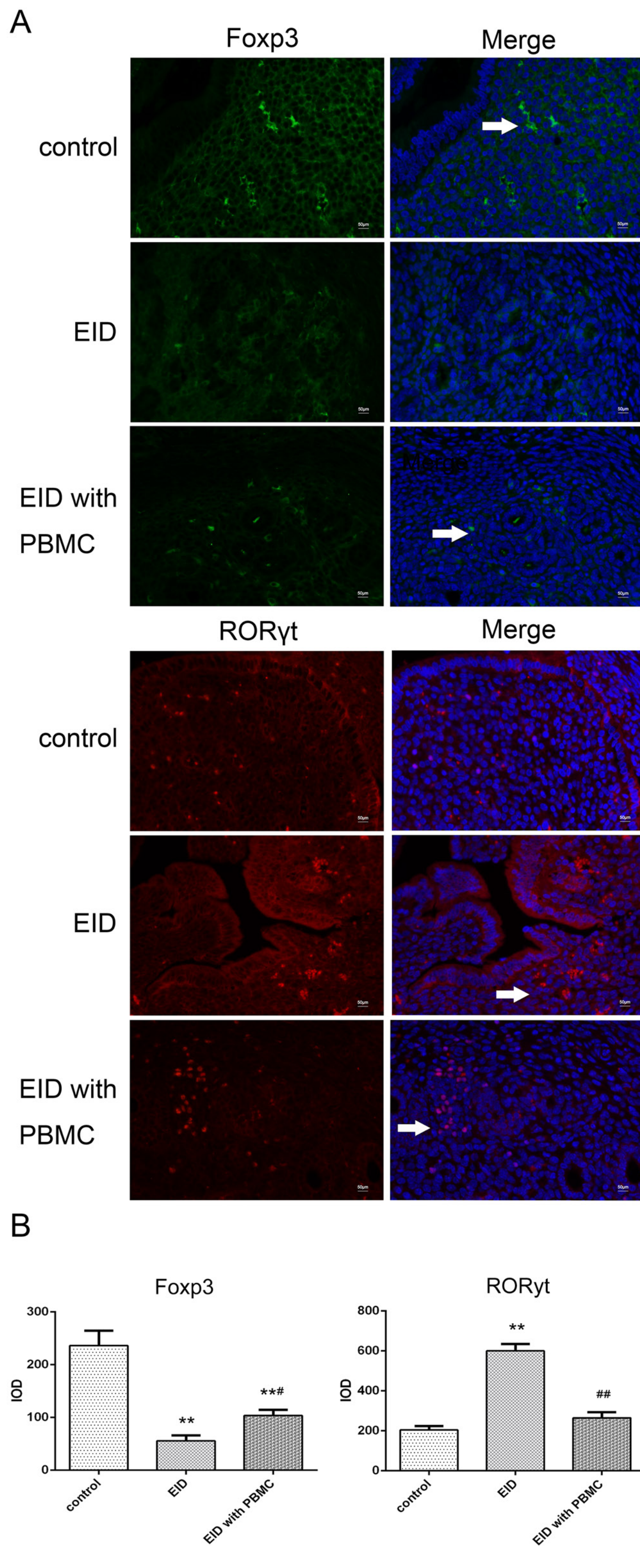

Fig. 4. Immunofluorescence analysis of Foxp3 and RORγt in mouse uterus on Pd4.0. (A) Immunofluorescence staining in each group. Foxp3 and RORγt were stained with green and red fluorescence, respectively and the nuclei were counterstained blue. The white triangle marks the protein expression of Foxp3 and the white arrow marks the protein expression of RORγt (bar = 50 μm). (B) Three fields were randomly selected from each slice in each group for analysis. Analysis of positive integrated optical density (IOD) using Image-pro Plus 6.0 software. The results are presented as the mean ± SD. ** $P < 0.01$ *vs*. control group, # $P < 0.05$ *vs*. EID group. ## $P < 0.01$ *vs*. EID group.

relative proportions of Treg and Th17 cells in the peripheral blood and spleen tissues did not show any significant differences among the groups. Plausible explanations include the following: i) Following intrauterine injection of PBMCs, majority of the immune cells enter directly into the uterine cavity, thereby possibly regulating the local endometrial immune microenvironment rather than systemic immunity. ii) The number of Treg cells in PBMCs may migrate to the fetal-maternal interface, while their secreted cytokines promote the immune balance of the local endometrium, thereby supporting embryonic implantation. iii) Supplementary PBMCs may trigger the necessary suitable initial inflammation for implantation.

As stated above, our results showed that the expression of Treg and Th17 cells related transcription factors and cytokines in the local endometrium changed after 2.5 days of PBMC administration. As naïve T cells take longer to proliferate and differentiate, it is conceivable that the earlier changes may be induced by the migration of systemic immune cells into the endometrium. In other similar studies, Wang *et al*. and Qin *et al*. demonstrated that intrauterine exogenous PBMC release cytokines (TGF-β and IL-2) and chemokines (CCL2, CCL17, CCL21, and CCL22), thereby regulating the intrauterine immune microenvironment and recruiting Treg cells into the endometrium [49, 50]. In addition, studies have found that trophoblast cells recruit Tregs to the endometrium through the following pathways: 1) Trophoblast cells secrete the chemokine CXCL16 [51]. 2) Human chorionic gonadotropin (HCG) produced by trophoblast cells directly recruit Tregs to the endometrium [52]. 3) HCG produced by trophoblast cells induce the production of chemokine CCL2 to recruit Tregs [53]. Based on the above results, we propose the following hypothesis: An endometrial scratching reaction may be produced during the intrauterine injection. The healing and repair process of the scratch causes a significant increase in macrophages, dendritic cells, and pro-inflammatory cytokines, which exert a positive effect on implantation [54, 55]. We speculate that approximately 48 h later, the exogenous PBMCs may release cytokines (TGF-β and IL-2) and chemokines (CCL2, CCL17, CCL21, CCL22) to regulate the intrauterine immune microenvironment, and then recruit Treg cells to the endometrium. At this time, the inflammatory response is weakened and immune tolerance is enhanced, which is conducive to endometrial receptivity and embryo implantation. However, the precise mechanism needs to be confirmed by further research.

In the past 20 years, several studies have demonstrated an improvement in embryo implantation following regulation of the immune status of the endometrium. Takabatake *et al*. (1997) and Fujita *et al*. (1998) demonstrated that administration of splenocytes or thymocytes, respectively, into the endometrium resulted in embryo implantation in mice [56, 57]. Subsequent studies that investigated the effect of intrauterine administration of PBMCs have shown that it effectively improves pregnancy rates in patients with RIF following ET [12–14, 21]. Moreover, Ideta *et al*. demonstrated that intrauterine administration of PBMCs prior to bovine ET improved the implantation rate. The authors of that study examined gene expression levels in PBMCs and lymphoid cells from uterine lumen and suggested that IL-1, IL-8 and macrophage-colony stimulating factor were more abundant in the PBMC group [58, 59] In addition, Fujiwara *et al*. found that PBMCs from pregnant women increased the secretion of progesterone and promoted embryo invasion *in vitro* [60]. In recent years, several clinical studies have confirmed the beneficial effects of intrauterine administration of PBMCs, highlighting its application prospects for RIF [61]. However, no study has explored the effects of PBMCs on immune cell balance. Therefore, our team is the first to study the balance between Treg and Th17 cells in intrauterine implantation

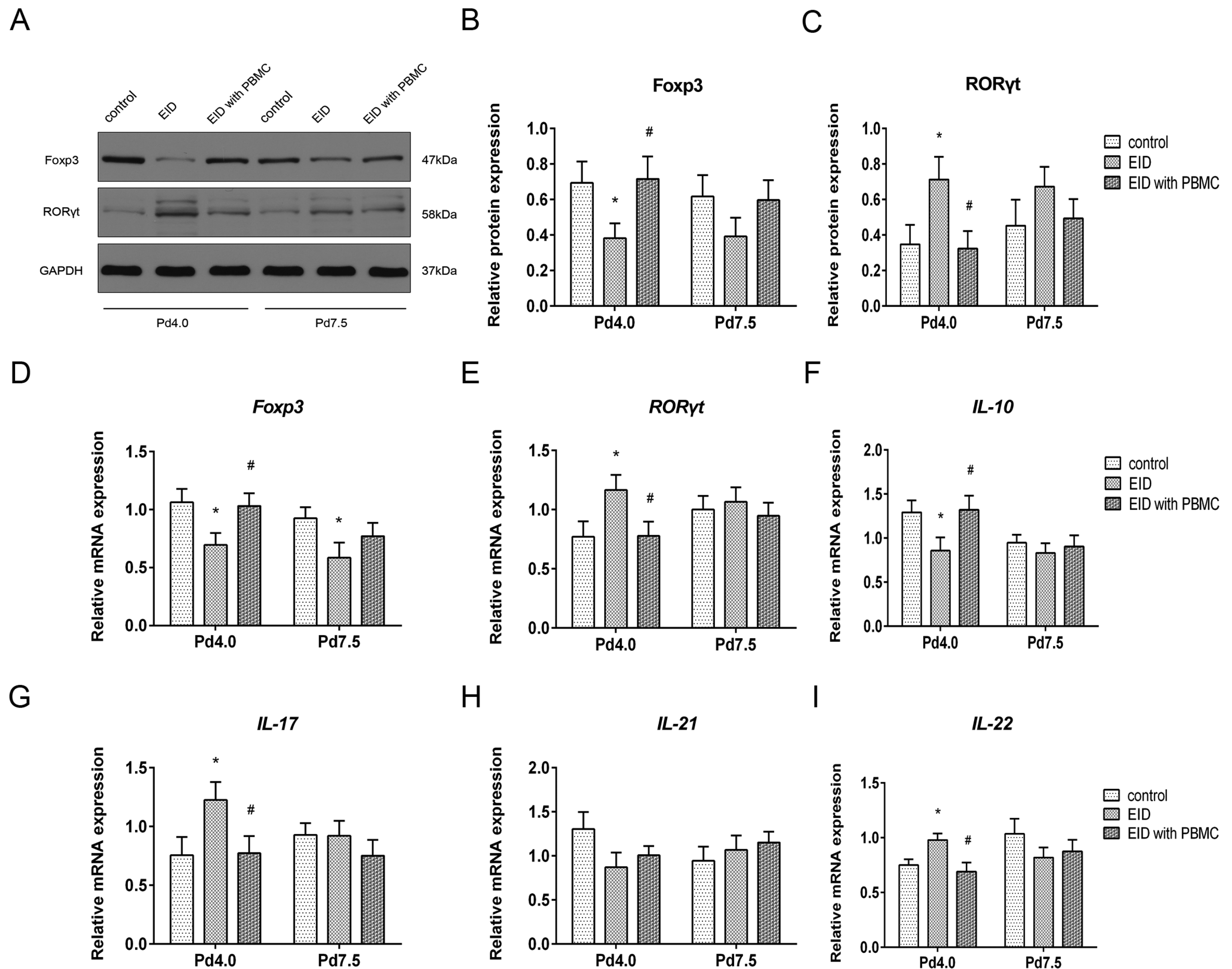

Fig. 5. Expression of transcriptional factors and cytokines involved in Treg and Th17 cell differentiation and function in mouse uterus on Pd4.0 and Pd7.5. Twelve mice were tested in each group. (A–C) Protein levels of Foxp3 and RORγt were determined by western blotting. (D–I) mRNA levels of *Foxp3*, *RORγt*, *IL-10*, *IL-17*, *IL-21*, and *IL-22* were determined by RT-PCR. The data are presented as the mean ± SEM. * P < 0.05 *vs*. control group, # P < 0.05 *vs*. EID group.

sites. The present study demonstrates for the first time that intrauterine administration of PBMCs may alter the balance between Treg and Th17 cells in the uterus. These results offer a novel insight into the underlying mechanisms of the implantation-promoting effects of PBMCs. Furthermore, these results provide theoretical support for the potential clinical application of PBMCs in patients with RIF.

However, the present study has certain limitations. The fate of the PBMCs following entry into the uterine cavity was not tracked or recorded. We only observed the effects of intrauterine administration of PBMCs on local immunity, whereas their effects on peripheral and local immune balance in the second and third trimesters of pregnancy were not explored. Moreover, the in-depth mechanism of how intrauterine administration of PBMCs improves the local balance of Treg/Th17 cells is not fully understood, and will be investigated in futurer studies.

Conflict of interests: The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81701530 and 81701476), the National Major Science and Technology Project of China (2017ZX09304022), and the Natural Science Foundation of Hubei Province (2017CFB626).

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