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Pharmacological activation of rev-erba suppresses LPS-induced macrophage M1 polarization and prevents pregnancy loss

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Abstract

Background: Circadian rhythm is an important player for reproduction. Rev-erba, a significant clock gene, is involved in regulating cell differentiation, inflammation and metabolism. Macrophage polarization plays crucial roles in immune tolerance at the maternal-fetus interface, which also modulates the initiation and resolution of inflammation. Alteration of macrophage polarization induces adverse pregnancy outcomes such as infertility, recurrent spontaneous abortion and preterm labor.

Results: Decidual macrophages from LPS-induced mice abortion model displayed M1-like bias, accompanied by decreased expression of Rev-erba. SR9009, an agonist of Rev-erba, may reduce lipopolysaccharide (LPS)-induced M1 polarization of macrophages via activation of PI3K but not NF-κB signaling pathway. Furthermore, SR9009 could reduce M1-like polarization of decidual macrophages induced by LPS and attenuate LPS-induced resorption rates in mice model.

Conclusions: Both in vivo and in vitro experiments demonstrated that the pharmacological activation of Rev-erba using SR9009 could attenuate the effect of LPS on macrophage polarization and protect pregnancy. This study may provide a potential therapeutic strategy for miscarriage induced by inflammation.

Keywords: Rev-erba, Decidual macrophages, M1/M2 polarization, Pregnancy

Background

Circadian rhythm is an endogenous free running cycle lasting near 24 h. In mammals, suprachiasmatic nuclei (SCN) is a master pacemaker coordinating the environmental changes to physiological activities [1]. Meanwhile, SCN synchronizes activities of other nucleus and peripheral organs by directly synaptic transmission or secreting peptides [2]. In molecule level, the circadian rhythm is regulated by clock genes in a transcriptional-translational loop. Brain and muscle ARNT-like 1

(Bmal1) and circadian locomotor output cycles kaput (Clock) are two core clock genes. BMAL1-CLOCK heterodimers modulate the transcription of clock genes with E-box sequence such as Per1–3, Cry1–2, Rev-erba. In the transcriptional-translational feedback loop, Rev-erba is an important clock gene and its protein directly represses the transcription of Bmal1 [1, 3]. In addition, Rev-erba, as a transcription factor, is reported to be involved in regulation of behavior rhythm, metabolism, autophagy and inflammation [4–7]. Therefore, Rev-erba may be an important therapeutic target of multiple diseases.

Circadian rhythm plays crucial roles in reproduction. Shift work is a common form of circadian rhythm disruption. Epidemiological studies have demonstrated that shift work increased the risk of infertility, menstrual

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dysregulation and miscarriage [8, 9]. It has reported that knockout of clock genes such as *Bmal1*, *clock*, *per1* and *Rev-erba* in mice showed a series of adverse pregnancy outcomes like implantation failure and miscarriage [3, 10, 11]. Thus, disruption of circadian rhythm may be an important cause of adverse pregnancy outcomes. As is well known that intrauterine inflammation can destroy immunologic microenvironment and trigger spontaneous abortion and preterm birth [12]. Previous studies proved pharmacological activation of *Rev-erba* suppressed the inflammatory response [7]. Whether *Rev-erba* is involved in maintaining the balance of immunologic microenvironment by inflammatory regulation remains unclear.

During normal pregnancy, maternal immune cells take important parts in immune tolerance to semi-allogeneic fetus. Decidual macrophages (dMφs) are the second abundant immune cells next to natural killer (NK) cells in the decidua, and play roles in maintaining the balance of immunologic microenvironment at maternal-fetal interface [13]. DMφs are characterized by high plasticity, whose function can be altered on the basis of the different tissue microenvironment. Parallel to Th1/Th2 paradigm, macrophages are originally divided into classically activated (M1) and alternatively (M2) populations according to their function and production of cytokines [13, 14]. M1 population is characterized by pro-inflammatory phenotype and generated in the exposure of pathogen such as LPS and pro-inflammatory cytokines like interferon- γ (IFN- γ) and tumor necrosis factor (TNF)- α . M1 population shows high expression of CD80, CD86, iNOS and is more effective at microbicidal properties and switching T-cell responses to Th1 immune response. M2 population exhibits anti-inflammation phenotype and is induced in the presence of interleukin (IL)-4, IL-13, or IL-10. M2 population is characterized by high expression of CD163, CD206, CD209 and Arg1, and plays roles in immunosuppression, tissue remodeling and promotion of immunomodulatory profile. Once the balance of M1/M2 populations is disrupted, the critical events of pregnancy like decidualization and vascular remodeling display pathological behaviors [13, 15]. Thus, the polarization of macrophage is significant for successful pregnancy. Circadian rhythm also exists in macrophages. The expression of cytokines in macrophages shows fluctuation of circadian rhythm and *Rev-erba* modulates the expression of some cytokines in macrophages [16–18]. However, whether *Rev-erba* modulates polarization of macrophage is undefined.

LPS is the primary constituent of the outer membrane of Gram-negative bacteria and has been regarded as an immune stimulatory molecule. It can elicit pro-inflammatory response in many cells such as macrophage and neutrophil [19]. Thus, it has been used to

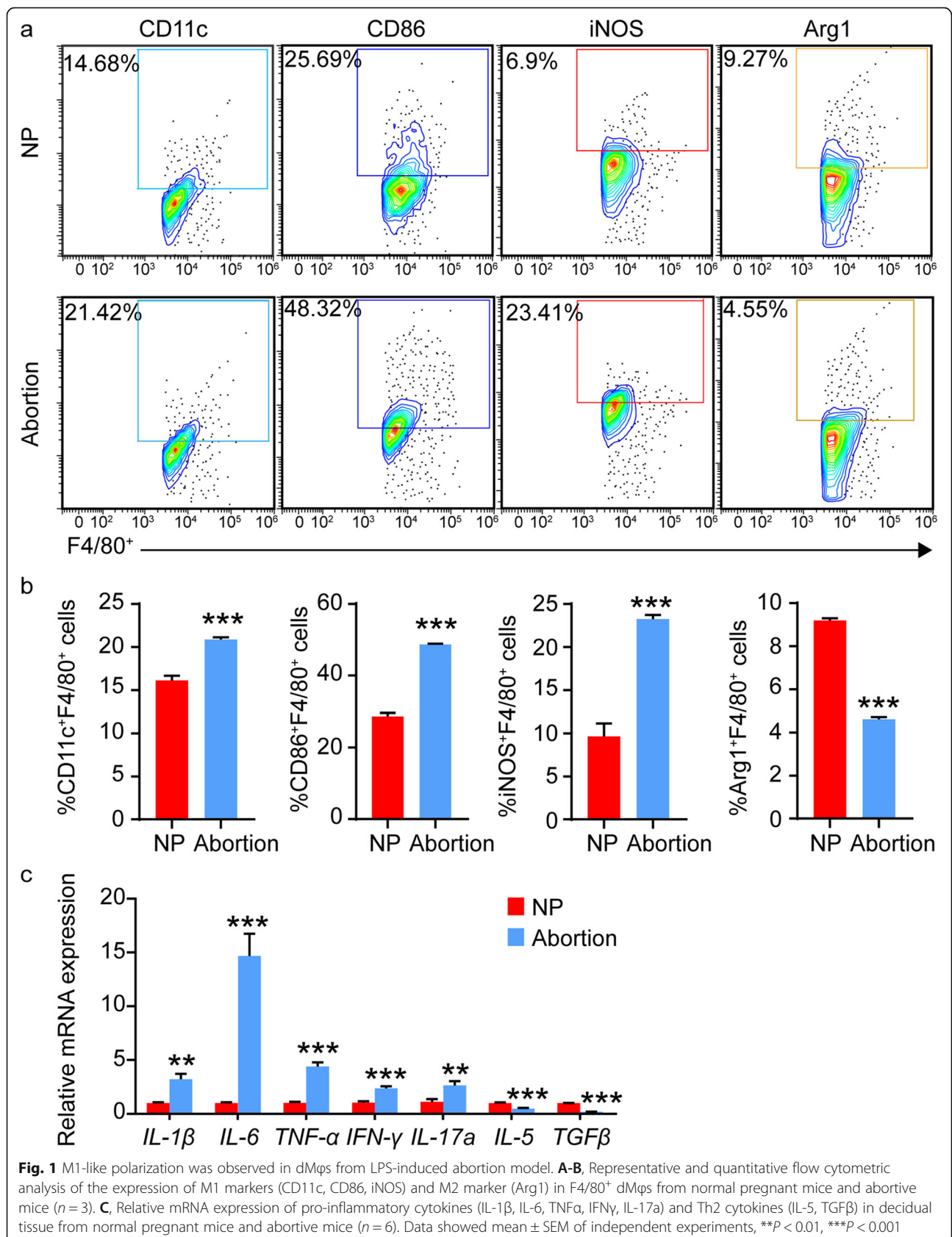
construct many inflammatory disease models like miscarriage and endometritis. Toll-like receptor (TLR) 4, a member of TLR family, recognizes the microbe-associated molecular patterns, including LPS [20]. The activation of TLR4 triggers the activation of NF- κ B signaling pathway by recruiting downstream adaptors and then induces the expression of inflammatory cytokines. Previous studies proved LPS suppressed the expression of clock genes including *Rev-erba* [7, 21]. Moreover, pharmacological activation of *Rev-erba* could suppress LPS-induced inflammatory response [21]. Therefore, there may be an interaction between inflammation induced by LPS and *Rev-erba*. But whether *Rev-erba* alters the changes of macrophages induced by LPS and attenuates the effect of inflammation on pregnancy remain unclear.

In this study, we observed the phenotype of dMφs and the expression of *Rev-erba* in dMφs from mice in LPS-induced abortion model. Then, we analyzed the phenotype changes of differentiated macrophages from U937 under the stimulation of LPS and the effect of SR9009, an agonist of *Rev-erba*, on phenotype changes of differentiated macrophages from U937 treated with LPS. Moreover, the protective role of SR9009 on abortion induced by LPS was explored. Our study may provide some novel strategies for miscarriage induced by inflammation.

Results

M1-like polarization and downregulated expression of *rev-erba* in dMφs were observed in LPS-induced mice abortion model

LPS has been widely used to establish various animal models, such as inflammatory diseases and spontaneous abortion [22, 23]. To investigate the effect of LPS on polarization of dMφs in mice, pregnant mice were injected with LPS on E7.5. As shown in Fig. 1A-B, the expression of M1 makers (CD11c, CD86 and iNOS) was increased, whereas the expression of M2 markers (Arg1) was decreased in F4/80⁺ dMφs from LPS-induced mice abortion model compared with those from control mice. LPS promoted the secretion of pro-inflammatory cytokines in macrophages [24]. We also detected the mRNA level of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α , IFN- γ , IL-17a) was significantly upregulated in decidual tissue from LPS-treated mice than those in control mice (Fig. 1C). Additionally, the mRNA level of Th2 cytokines (IL-5, TGF β) was significantly downregulated in decidual tissue from LPS-treated mice than those in control mice (Fig. 1C). Consistent with our previous studies that LPS treatment in vitro suppress the expression of *Rev-erba* in human endometrial stroma cells (ESCs) [21], the expression of *Rev-erba* in decidual tissue from mice treated with LPS was dramatically downregulated



(Fig.2A). The immunofluorescence staining further proved that LPS administration significantly decreased the expression of Rev-erba in F4/80⁺ dMφs of pregnant mice (Fig.2B-C).

Activation of rev-erba reduced the effect of LPS on macrophage polarization

Excessive inflammation due to bacterial infection is a common cause of early pregnancy loss [25]. LPS, a component of Gram-negative bacteria, induced M1-like polarization of dMφs and decreased the expression of Rev-erba in dMφs from mice abortion model. We wondered if LPS treatment could also affect the expression of Rev-erba and phenotype changes in differentiated macrophages from U937. As shown in Fig. 3A-E, LPS administration significantly decreased the expression of Rev-erba in differentiated macrophages from U937 with M1 dominance. We then investigate if activation of Rev-erba could reduce LPS-induced M1 polarization of differentiated macrophages from U937. The results in Fig. 3B-E demonstrated that administration of SR9009 could reduce the expression of M1 marker (CD86) and increase the expression of M2 markers (CD163, CD206 and CD209), suggesting that activation of Rev-erba alleviated LPS-induced M1 polarization of differentiated macrophages from U937. We then further explore the

potential downstream signaling pathway involved in the regulation. Previous studies proved PI3K/Akt signaling pathway participated in macrophage polarization [26, 27]. To prove Rev-erba might regulate phenotype of macrophages via PI3K signaling pathway, we used LY294002, a PI3K inhibitor, to block PI3K signaling pathway and detected the phenotype changes of differentiated macrophages from U937. We found that inhibition of PI3K by LY294002 suppressed the effect of SR9009 on LPS-induced M1 polarization, suggesting that the PI3K signaling pathway may be involved in the regulation of Rev-erba on LPS-induced M1 polarization (Fig.3B-E). We have previously reported that LPS increased the expression of TLR4 and then recruited downstream molecules to activate the NF-κB signaling pathway and SR9009 suppressed the activation of NF-κB induced by LPS in uterine endometrial stromal cells [21]. Unexpected, SR9009 could not repress the increased expression of TLR4 and NF-κB induced by LPS in differentiated macrophages from U937 (Fig.3F). In addition, SR9009 inhibited LPS-induced ROS production in differentiated macrophages from U937, which could also be reversed by the PI3K inhibitor (Fig.3G). Thus, SR9009 may attenuate the M1 polarization induced by LPS treatment via the PI3K signaling pathway but not the NF-κB signaling pathway.

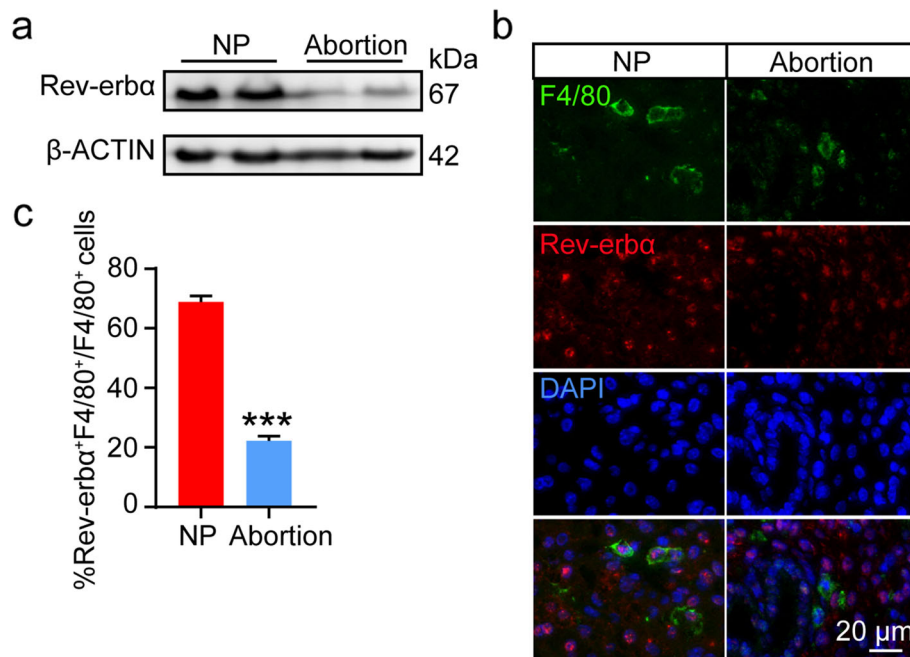
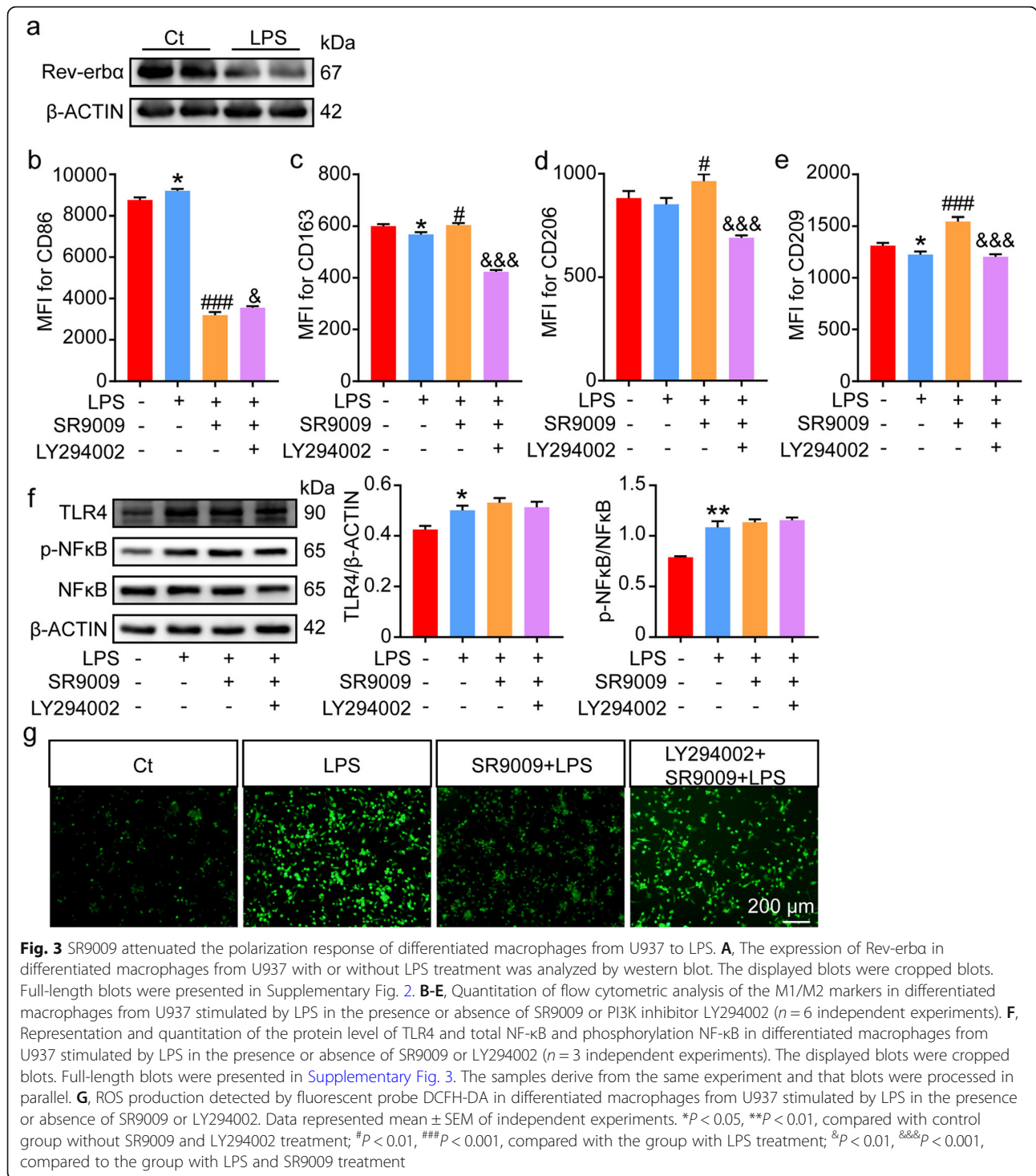


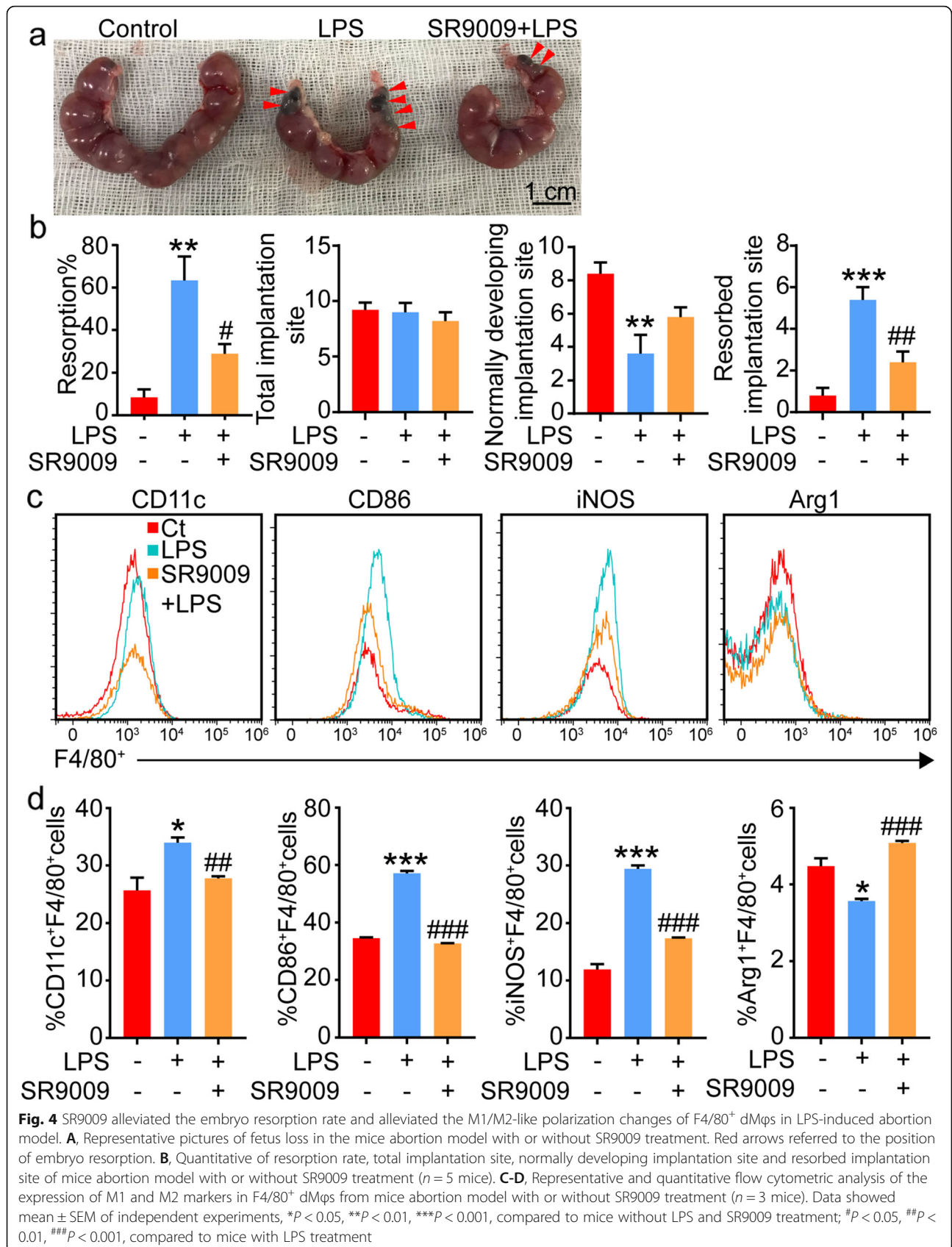
Fig. 2 Downregulated expression of Rev-erba was observed in dMφs from LPS-induced abortion model. **A**, Protein level of Rev-erba in decidua tissue from normal pregnant mice and abortive mice was analyzed by western blot. The displayed blots were cropped blots. Full-length blots were presented in Supplementary Fig. 1. **B**, Expression of Rev-erba in F4/80⁺ dMφs from mice with LPS treatment was analyzed by immunofluorescence. **C**, The portion of F4/80 and Rev-erb double-positive cells among the total F4/80 positive cells ($n = 13$ images from 3 mice). Data showed mean \pm SEM of independent experiments. *** $P < 0.001$



Activation of rev-erba rebalanced M1/M2 polarization of dMφs and alleviated the abortion rate induced by LPS

The pharmacological activation of Rev-erba attenuated the effect of LPS on decidual M1/M2 polarization. Whether activation of Rev-erba prevents LPS-induced pregnant loss needs to be further confirmed. Figure 4A-B showed that SR9009 significantly attenuated the

embryo resorption rate induced by LPS. Consistent with the results in vitro, the expression of M1 markers (CD11c, CD86 and iNOS) was decreased in dMφs from mice treated with LPS/SR9009 compared to those from mice treated with LPS alone. In contrast, the expression of M2 marker (Arg1) was increased in dMφs from mice treated with LPS/SR9009 compared to those from mice



treated with LPS alone (Fig. 4C-D). Therefore, the pharmacological activation of Rev-erb α reduced pregnancy loss induced by LPS, accompanied by the rebalance of M1/M2 polarization of dM ϕ s.

Discussion

Successful pregnancy depends on harmonious micro-environment at the maternal-fetal interface [28, 29], where multiple immune cells such as NK cells, macrophages and T cells with unique phenotypes are present to protect but not attack fetus. Macrophages, as the second abundant immune cells, play important roles in critical biological events of pregnancy establishment and maintenance [13, 30]. Macrophages display plasticity with different M1/M2 polarization states during distinct pregnancy period. Higher M1/M2 ratio of dM ϕ s is observed in recurrent spontaneous abortion compared with that in normal pregnancy [31]. In this study, we demonstrated that decreased expression of Rev-erb α and M1 polarization in dM ϕ were displayed in LPS-treated pregnant mice, which was also accompanied by increased embryo resorption rate in early pregnancy. These results suggested that the decreased expression of Rev-erb α might be an important trigger in LPS-induced miscarriage and M1 polarization.

Miscarriage is one of the common adverse pregnancy outcomes and approximately accounts for one in five pregnancies. It has been proved up to 15% of early miscarriages and 60% of late miscarriages were attributed to infection [25]. Many pathogens like bacteria, protozoa and virus can trigger infection, leading to miscarriage [32, 33]. Bacterial infection can initiate the response of innate immune system. Macrophages play crucial roles in the initiation and resolution of inflammation, which modulate their phenotype in response to environmental signals such as LPS from invading bacteria [34]. LPS is a primary infectious stimulus, which can induce inflammatory response. Recent researches have proved LPS is associated with embryonic abortion and implantation failure in mouse model. Macrophages treated with LPS secrete pro-inflammatory cytokines [19]. During implantation process, trophoblast invasion induces damage of maternal tissue and cell apoptosis, which promoting inflammatory response. However, excessive inflammatory response could induce pregnancy loss such as spontaneous abortion. We also showed that the expression of IFN- γ and TNF- α were dramatically increased in decidual tissues from LPS-induced abortion model.

Disruption of circadian rhythm can induce adverse pregnancy outcomes [35]. SCN, as a core pacemaker, transmitted the light entrained rhythm to other nucleus and regulated the secretion of hormones in ovary by hypothalamus-pituitary-ovary axis [36]. Some functions of uterine are regulated by hormone secreted by ovary.

Once the disruption of sleeping, the circadian rhythm in organism is broken and most functions of cells are abnormal [37]. In molecular level, circadian rhythm in cells is modulated by their own transcriptional-translational loop consisted of clock genes. It has been reported that knockout of clock gene such as Bmal1, Clock, Per1 can induce abnormal pregnancy outcomes [3, 10]. Rev-erb α , as an important clock gene in transcriptional-translational loop, regulates inflammation, autophagy and metabolism, apart from modulating circadian rhythm. Rev-erb α is reported to inactivate cancer markers, proposing that it could be a potential strategy for cancer treatment [38]. In this study, we demonstrated the expression of Rev-erb α was decreased in both dM ϕ s and differentiated macrophages from U937 after LPS treatment. Moreover, it has been demonstrated that knockdown of Rev-erb α induced pro-inflammatory response in ESCs [21]. Whether the downregulation of Rev-erb α can strengthen the sensitivity to pathogen stimulation need to be proved in the future.

LPS can trigger the activation of TLR4 and NF- κ B signaling pathway. Moreover, upregulation of Rev-erb α can repress the activation of NF- κ B signaling pathway induced by LPS in ESCs [21]. Unexpected, this mechanism was not involved in the regulation of Rev-erb α on macrophage polarization. However, PI3K inhibitor can alleviate the role of SR9009 on polarization of differentiated macrophages from U937 with LPS treatment, suggesting PI3k/AKT might be important in the attenuation of LPS-induced decidual M1 polarization. In addition, we suspected the function of other cells which are expressing TLR4 in the implantation site may be disturbed by LPS, and then these cells induced phenotype changes of dM ϕ s, which aggravated the adverse pregnant outcomes. That is to say, the phenotype changes of decidual macrophage may be affected not only directly by LPS but also indirectly by other cells influenced by LPS. In order to confirm the role of decidual macrophages with decreased expression of Rev-erb α on pregnant outcomes, we will construct Rev-erb α -conditional-knockout mice to knock out Rev-erb α in macrophages of mice in the future.

The anti-inflammation of Rev-erb α indicated that it could be a potential target for prevention of abortion induced by inflammation. Indeed, the activation of Rev-erb α decreased LPS-induced embryo resorption rates of pregnant mice. Although SR9009 also alleviated the changes of M1/M2 polarization of dM ϕ s from LPS-treated mice, this is not the only mechanism of SR9009 in prevention of pregnancy failure. There are many cell types like decidual stromal cells, NK cells and T cells in decidual tissues. LPS can also change the function of these cells, but whether pharmacological activation of Rev-erb α reduce the functional changes of these cells

with LPS treatment need to be further determined in our future study.

In summary, LPS induced inflammatory response and promoted M1-like polarization of macrophages in decidua of mice. Meanwhile, LPS repressed the expression of Rev-erb α in macrophages. Pharmacological activation of Rev-erb α using SR9009 may repress M1 polarization of differentiated macrophages from U937 induced by LPS via PI3K signaling pathway. In vivo, SR9009 attenuated LPS-induced abortion rate in mice, and reduced the M1 polarization in macrophages. Our study might supply a potential target for the recurrent spontaneous abortion, especially for inflammation-related miscarriage.

Methods

Mice and LPS-induced abortion model

The male and female C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. All mice were bred in room of 22–25 °C, 40–60% relative humidity, 12 h light-12 h dark cycles. All mice experimental procedures were approved by Institutional Animal Care and Use Committee at Fudan University. The female mice were mated with male mice at 19:00. By 7:00 next morning, vaginal plug was detected and referred as day 0.5 of embryos (E0.5). For abortion model, all the mice were intraperitoneally injected with 0.25 mg/kg LPS at E7.5. To evaluate the effect of SR9009 on LPS-induced abortion, pregnant female mice were divided into two groups. They were administrated respectively with 50 mg/kg SR9009 or corn oil by intraperitoneal injection at 16:00 (the time point of peak expression of Rev-erb α) on E6.5 once daily for 5 days. All mice were sacrificed on E13.5 to analyze the abortion rates. All measurements were conducted blind to the group.

Flow cytometry

In order to obtain single cell suspension, the adherent cells were digested by 0.25% trypsin with 0.02% EDTA (Genom, Shanghai, China), and the tissues were digested with Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) containing 1.0 mg/ml collagenase IV (Sigma-Aldrich, MO, USA) and 150 U/ml DNase I (Sigma-Aldrich, MO, USA). The expression of cell-surface and intracellular molecules was detected by flow cytometry. For cell-surface molecules, the cells were incubated with following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80; PerCP/Cyanine5.5 anti-mouse CD11c; Brilliant Violet (BV) 421-conjugated anti-mouse CD86; (phycoerythrin (PE)-conjugated anti-human CD163; allophycocyanin (APC)-conjugated anti-human CD206; PE/Cyanine7 anti-human CD86; PerCP/Cyanine5.5 anti-human CD209 (Biolegend, CA, USA). For intracellular molecules tests, the cells were fixed and permeabilized with Fix/Perm Kit (Biolegend, CA, USA) and then

incubated with antibodies: APC-eFluor 780-conjugated anti-mouse iNOS (Ebioscience, CA, USA); PE-conjugated anti-mouse Arg1 (Invitrogen, MA, USA). A minimum of 10,000 events were collected by a BD or Beckman flow cytometer and analyzed with FlowJo or CytExpert software.

Quantitative real-time polymerase chain reaction (qPCR)

The RNA in adherent cells or tissues was extracted by TRIzol reagent (Takara, Honshu, Japan) according to manufacturer's instruction. 1 μ g RNA was reverse-transcribed into complementary DNA (cDNA), which was amplified with SYBR Green PCR Master Mix (Takara, Honshu, Japan) on ABI PRISM 7900 Sequence Detection System (Applied Biosystems, MA, USA). β -actin (Actb) was used as an internal control to normalize relative changes in gene expression through $2^{-\Delta\Delta C_t}$ method. The specific primers were as follows: IL-1 β , forward 5'- AATGCCACCTTTTGACAGTGATG-3' and reverse 5'- AGCTTCTCCACAGCCACAAT-3'; IL-6, forward 5'- ATC-CAGTTGCCTTCTTGGGACTGA-3' and reverse 5'- TAAGCCTCCGACTTGTGAAGTGGT-3'; TNF- α , forward 5'- AGGGTCTGGGCCATAGAACT-3' and reverse 5'- CCACCACGCTCTTCTGTCTAC-3'; IFN- γ , forward 5'- GCTACACACTGCATCTTGGC - 3' and reverse 5'- CATGTCACCATCCTTTTGCCAG-3'; IL-17a, forward 5'- TTAACTCCCTTGCGCAAAA - 3' and reverse 5'- CTTTCCCTCCGCATTGACAC -3'; IL-5, forward 5'- CTCTGTTGACAAGCAATGAGACG - 3' and reverse 5'- TCTTCAGTATGTCTAGCCCCTG - 3'; TGF β , forward 5'- CTCCCGTGGCTTCTAGTGC - 3' and reverse 5'- GCCTTAGTTTGGACAGGATCTG - 3'.

Western blot assay

The whole proteins were extracted from homogenized cells and tissues using radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) containing phosphatase repressor (Roche, Basel, Switzerland) and protease inhibitor (Beyotime, Shanghai, China) based on previous publication [21]. Samples containing 20 μ g proteins were separated by 10% sodium dodecyl sulfonate polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany) and then incubated with primary antibodies (anti-Rev-erb α (Santa Cruze, TX, USA); anti-TLR4 (Abcam, CA, USA); anti-p-NF- κ B (Cell Signaling Technology, MA, USA); anti-NF- κ B (Cell Signaling Technology, MA, USA); anti- β -ACTIN (Abcam, CA, USA)) after blocked with 5% non-fat dry milk powder in tris-buffered saline (TBS) with 0.1% Tween 20 (TBST). The membrane was washed with TBST for 4 times (5 min at every turn) and incubated with secondary antibodies. The membrane was visualized by an enhanced chemiluminescence detection system. β -ACTIN was used to

normalize the protein expression by greyscale analysis using ImageJ software.

Immunofluorescence

Mouse decidual tissues were fixed using paraformaldehyde and then prepared for paraffin section. Paraffin sections was applied with citrate sodium solution for antigen retrieval after dewaxed using dimethylbenzene and ethanol with different concentrations. The slices were sealed with 0.05% TritonX-100 and 10% donkey serum and then incubated with primary antibodies (anti-F4/80 (Invitrogen, MA, USA); anti-Rev-erb α (Santa Cruze, TX, USA)) for overnight at 4 °C. After washed by TBS for three times, the slices were incubated with secondary antibodies for 2 h at room temperature. Next, the slices were stained with 4',6-diamidino-2-phenylindole (DAPI) for 7 min and then washed with TBS for 3 times. At last, the slices were sealed with mounting medium and photographed using a fluorescence microscope.

Cell culture and treatment

U937 cells, human monocyte cell line, were cultured with complete medium (RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin (Sangon Biotech, Shanghai, China) in a 37 °C humidified incubator containing 5% CO₂. To obtain differentiated macrophages from U937, U937 cells were dealt with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, MO, USA) for 24 h. The polarization status of differentiated macrophages from U937 were analyzed after 100 ng/ml LPS (Sigma-Aldrich, MO, USA) treatment for 48 h. For analysis of Rev-erb α activation, differentiated macrophages from U937 were dealt with 10 μ M SR9009 for 4 h before LPS treatment. For signal pathway analysis, differentiated macrophages from U937 were treated with LY294002 (MedChemExpress, NJ, USA) for 1 h before SR9009 treatment.

Reactive oxygen species

The treated cells dealt with serum-free RPMI1640 medium supplemented with 1 μ M 2',7'-dichloro-4,6-diamidino-2-phenylindole (DCFH-DA) (Sigma-Aldrich, MO, USA) for 30 min at 37 °C. And then the cells were washed three times with serum-free RPMI1640 medium. The cells were taken pictures using a fluorescence microscope.

Statistical analysis

All statistics were assessed by GraphPad Prism Version 7 and were presented as mean \pm standard error of the mean (SEM). Comparison between the two groups was analyzed by Student's t-test. Multiple groups were analyzed by ANOVA. $P < 0.05$ was defined as statistically significant difference.

Abbreviations

LPS: lipopolysaccharide; SCN: suprachiasmatic nuclei; Bmal1: Brain and muscle ARNT-like 1; Clock: circadian locomotor output cycles kaput; dM ϕ s: decidual macrophages; NK: natural killer; IFN- γ : interferon- γ ; TNF- α : tumor necrosis factor- α ; IL: interleukin; TLR: Toll-like receptor; ESCs: endometrial stroma cells; SEM: standard error of the mean; qPCR: quantitative real-time polymerase chain reaction; TBS: tris-buffered saline; DAPI: 4',6-diamidino-2-phenylindole; FBS: fetal bovine serum; DCFH-DA: 2',7'-dichloro-4,6-diamidino-2-phenylindole diacetate

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12865-021-00438-4>.

Additional file 1: Fig. S1. Uncropped full-length blots with high contrast and low contrast were included for Fig. 2A.

Additional file 2: Fig. S2. Uncropped full-length blots with high contrast and low contrast were included for Fig. 3A.

Additional file 3: Fig. S3. Uncropped full-length blots with high contrast and low contrast were included for Fig. 3F. The samples derive from the same experiment with and that blots were processed in parallel. *The blot was not shown in Fig. 3F.

Acknowledgments

Not applicable.

Authors' contributions

L.C. designed this project, performed experiments, analyzed data and drafted the manuscript. F.X. performed experiments. S.W. helped to analyze data. X.L. and H.L. contributed to performed experiments. Y.D. took part in discussing this project. M.D. conceived this project and revised this manuscript. All authors reviewed and approved the final manuscript.

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Availability of data and materials

The datasets analyzed in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study is reported in accordance with ARRIVE guidelines. All animal protocols were approved by Institutional Animal Care and Use Committee at Fudan University, the ethics committee approval code was FE20053. This study does not involve the use of human data or tissue. All experiments were performed in accordance with relevant guidelines and regulations at Obstetrics and Gynecology Hospital of Fudan University. All mice were sacrificed by exsanguination under anesthesia by 5% isoflurane. The sacrificed mice were handed over to special departments for unified treatment. All procedures were approved by Institutional Animal Care and Use Committee at Fudan University.

Consent for publication

Not applicable.

Competing interests

All authors have not any conflicts of interest to declare.

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