# Anti-inflammatory effects of Placenta-derived exosomal miR-24-1-5p through targeting TNFAIP8 and its implication in enhancing placental endotoxin tolerance in a model of lipopolysaccharide (LPS)-induced placental inflammation

**Yongqing Zhang**
Women's Hospital, School of Medicine, Zhejiang University
**Lejun Li**
Women's Hospital, School of Medicine, Zhejiang University
**Luping Chen**
Women's Hospital, School of Medicine, Zhejiang University
**Tiantian Fu**
Women's Hospital, School of Medicine, Zhejiang University
**Guohui Yan**
Women's Hospital, School of Medicine, Zhejiang University
**Zhaoxia Liang**
Women's Hospital, School of Medicine, Zhejiang University
**Meihua Sui**
Zhejiang University School of Medicine
**Danqing Chen** ( Chendq@zju.edu.cn )
Women's Hospital, School of Medicine, Zhejiang University

**Research Article**

**Keywords:** Intra-amniotic infection, Placental endotoxin tolerance, Placenta-derived exosomes, miR-24-1-5p, Tumor necrosis factor alpha-induced protein 8

**Posted Date:** August 11th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-3239227/v1

## Abstract

# Objective and design:

Intra-amniotic infection (IAI) represents a potentially catastrophic complication during parturition, with potential for significant maternal and neonatal consequences. As a crucial immunological organ at the maternal-fetal interface, the immunoregulatory capacities of the placenta and the underlying molecular dynamics at play in the pathogenesis of IAI continue to be unclear. Recent studies have found that placenta-derived exosomes (Pd-Exos) and their contained microRNAs (miRNAs) may be involved in immune regulation during pregnancy. This study aimed to investigate the immunoregulatory function of the placenta in connection with the progression of IAI, as well as to elucidate its potential molecular mechanisms.

# Material or subjects:

An IAI model was developed by co-culturing full-term placental explants obtained from elective cesarean sections with lipopolysaccharide (LPS). Using RT-qPCR and ELISA, the dynamic expression patterns of inflammatory factors within the placental explants were identified at varying time points post-LPS exposure. Pd-Exos were then isolated from the culture supernatant of placental explants and subjected to miRNA sequencing to pinpoint exosomal miRNAs integral to the immunoregulatory functions of the placenta. Subsequently, the role of a differentially expressed exosomal miRNA was validated.

# Results

In the early phase of LPS stimulation, extensive pro-inflammatory responses were observed in placental explants, evidenced by the augmented expression of TNF-α and IL-1β. However, as the LPS stimulation progressed to the latter stages, a decrease in the pro-inflammatory response was noted, alongside a gradually surging anti-inflammatory response, signified by a diminishing ratio between pro-inflammatory and anti-inflammatory indicators (TNF-α/IL-10 and IL-1β/IL-1Ra). Following prolonged LPS stimulation of placental explants, the expression of miR-24-1-5p was upregulated in Pd-Exos. Upon internalization by receptor cells (THP-1 cells and Swan 71 cells), miR-24-1-5p can inhibit the expression of its target gene Tumor necrosis factor alpha-induced protein 8 (TNFAIP8), thereby suppressing the expression of downstream inflammatory factors TNF-α and IL-1β.

# Conclusion

Prolonged exposure to the LPS in human term placental tissues induced endotoxin tolerance. Additionally, the placenta-derived exosomal miR-24-1-5p down-regulated the expression of the

inflammatory markers TNF-α and IL-1β by inhibiting the functionality of TNFAIP8, thereby contributing to the placental endotoxin tolerance.

# Introduction

Intra-amniotic infection (IAI) represents one of the severe complications associated with childbirth. The reported incidence ranges from 7.2–11.3%, making it a prevalent complication that can detrimentally impact maternal and child health [1–2]. IAI can instigate acute neonatal complications including neonatal pneumonia, meningitis, and septicaemia, and in extreme circumstances, can lead to neonatal mortality. For women, IAI can cause weak uterine contractions and secondary postpartum haemorrhage, which, in severe cases, can lead to maternal death [3].

Currently, risk factors for IAI include low parity, multiple vaginal examinations, use of intrauterine cardiac monitoring, meconium-stained amniotic fluid, and the presence of certain genital tract pathogens in the vagina [3–5]. IAI is predominantly attributed to the ascending infection of normal vaginal flora, however, the hematogenous spread of microorganisms also plays a significant role [6]. Studies have shown that the placenta, as an important immune organ at the mother-fetus interface, dispatches a variety of hormones and signaling substances that have been confirmed to participate in immune regulation during pregnancy [7–8]. However, the role of the placenta in the development of IAI remains ambiguous (does it mediate inflammatory signals, or does it suppress inflammation?).

The etiology of IAI is complex and highly heterogeneous. It can be caused by bacteria, viruses, mycoplasma, chlamydia, and may also be a mixed infection of multiple microorganisms [3]. This poses a significant challenge in studying the immunological role of the placenta during the occurrence of IAI by comparing placental tissues from pregnant women who have experienced IAI with those from non-infected women. Therefore, establishing a model of placental explants affected by a singular infectious factor in the context of IAI becomes extremely important. Escherichia coli is the most prevalent pathogen implicated in IAI, leading to severe adverse consequences for both mothers and infants [6]. Therefore, we have established an in vitro IAI model by co-culturing LPS with human placental explants. This set-up not only ensures the singularity of infectious factors in the model, it also allows for the controlled analysis of the interaction between LPS and placental explants over varying time frames. With this, we could investigate the temporal flux of placental inflammatory markers throughout different stages of infection.

microRNAs (miRNAs) are important cellular regulatory factors in immune responses, but there are few free circulating miRNAs in the extracellular environment, which are mainly packaged within Exosomes and act as a vehicles to transport miRNAs to target tissues or cells [9, 10]. Exosomes are small (30 ~ 150 nm diameter) membrane vesicles of endosomal origin that are continually secreted by a variety of cell types in response to physiological or pathological stimuli [11]. They have been demonstrated to exist in almost all biological fluids and have been found to contain various types of cargoes [12]. Furthermore, they can travel in the extracellular space as messengers carrying bioinformation in cell-to-cell

communication, and can also be internalized by target cells via membrane fusion or endocytosis. After subsequent release of their cargo, exosomes mediate various biological and pathological processes.

In this study, we initially established an in vitro IAI model by co-culturing placental explants with LPS, subsequently detecting the expression trend of inflammatory factors in these explants at various time points following LPS exposure. Furthermore, we elaborated on the roles of placenta-derived exosomes and their contained miRNAs in the placenta's immunomodulatory function during the IAI process. Our findings may reveal novel mechanisms and intervention strategies for IAI.

# Materials and methods

### Ethical approval

This study was conducted in accordance with *the Declaration of Helsinki* and was approved by the Ethics Committee of Women's Hospital, School of Medicine, Zhejiang University (No:*IRB-20211164-R*). Informed consent were obtained from all participating patients before the initiation of the study.

### Clinical sample collection and culture of placental explants

Upon acquiring informed consent from the patient, fresh placental tissues were obtained from full-term pregnancies ($38^{+0}$ to $41^{+6}$ weeks), specifically from women who underwent elective cesarean sections without any complications, within 0.5 hours of the procedure. The top 2mm segment, containing maternal decidua tissue, from the maternal aspect of the placenta was carefully dissected and discarded, and visible vascular tissue was also removed. Subsequently, the placental villous tissue was isolated, rinsed with sterile Phosphate-Buffered Saline (PBS) approximately 20 times, or until the point when no reddish fluid was observed to be discharged. Following thorough cleansing, proceed with the detailed dissection of the placental villous into explants weighing approximately 400 mg each, roughly the size of a soybean. Placental explants (10 grams), were cultured in medium (DMEM/F12) (Invitrogen (Carlsbad, CA, USA) supplemented with 2% exosome-depleted FBS (BI, Israel), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin, and subsequently incubated at an incubator at 37ºC and 5% $CO_2$. After 2 hours of stabilization, a slight precipitation of blood cells, instigated by the placental explants, was observed. These precipitates were then eliminated through an additional wash with sterile PBS, and the explants were subsequently incubated with a new medium.

Placental explants from identical maternal sources were classified into two groups for study: (1) Intra-Amniotic Infection (IAI) model group, wherein placental explants were co-cultured with a medium incorporating 1μg/mL or 10μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA); (2) The control group, consisting of placental explants from the same laboring woman, cultured merely in complete culture medium. Each group was subsequently subdivided into five equal parts, distinguished by incubation durations of 2h, 6h, 12h, 18h, and 24h, yielding fifteen portions in total. For culture, placental explants weighing 2 grams were positioned in individual wells of a 24-well culture plate, to which 2ml of medium was added. At the conclusion of their respective incubation periods, the placental explants and culture

media were collected and preserved at -80℃ until subsequent RT-qPCR and ELISA examinations were conducted. The clinical characteristics of 10 pregnant women used for placental explant collection are detailed in **Table S1**.

### Real-time quantitative PCR for cytokines and miRNAs

Total RNA was extracted from the tissues and cell lines employing the Trizol reagent (Takara Bio Inc., Otsu, Shiga, Japan), according to the guidelines provided by the manufacturer. Subsequent to isolation, the quality and quantity of the extracted RNA were thoroughly assessed. A total of 3 μg RNA was reverse transcribed into the complementary DNA (cDNA) using PrimeScript RT reagent Kit (Takara). Each 20 uL sample volume containing 10 μL of 2×TB Green PCR Master Mix (Takara), 250 nmol/L each specific primer, and 20 ng of cDNA was determined by qPCR. The specific primers used in qPCR (TNF-α IL-1β IL-10 IL-1Ra and GAPDH were shown in **Table S2**. Each cDNA sample was subjected to triplicate measurements during the qPCR process, and the resultant data was subsequently calculated by deriving the mean value from the triplicate outcomes. The relative expression levels of the target gene mRNA were quantified using the comparative Ct method, employing the 2^-ΔΔCt formula. GAPDH acted as the reference gene for normalizing variations in gene expression. miRNA quantification: The Bulge-loop$^{TM}$ RT primer and qPCR primers specific for miR-24-1-5p were designed and synthesized by RiboBio (Guangdong, Guangzhou, china). The U6 gene was used as an internal control. The primer sequences for the target gene TNFAIP8 of miR-24-1-5p are also provided in **Table S2**. All primers used in this study passed the validation test.

### Enzyme-linked immunosorbent assay (ELISA)

Post-culturing of placental explants via the aforementioned methods, we chosen explants that were cultured with 1μg/ml LPS for durations of 12h, 24h, 36h, and 48h to carry out ELISA and continually ascertain the levels of inflammatory factors in the supernatants. Contrastingly, explants cultured for identical durations solely in complete culture medium served as controls. The ELISA procedure hinged on commercially provided kits targeting TNF-α, IL-10, IL-1β (Absin Bioscience Inc., Shanghai, China) and IL-1Ra (Fine Biotech Co., Ltd, Wuhan, China), the usage of which complied strictly with the manufacturer's protocols. The quantification of the four inflammatory factors present in the supernatant of the placental explants was achieved by reading the optical density at 450nm. Each analysis was conducted in parallel with a known standard, and every treatment was repeated three times. The final concentration of the inflammatory factors was determined by calculating the average of these repetitions.

### Pd-Exos isolation and characterization

In alignment with the data obtained from the previously conducted RT-qPCR and ELISA analyses, we proceeded with the extraction of Pd-Exos from the supernatant cultured from placental explants for a period of 18h (utilizing complete medium or 1 μg/ml LPS) for further examination. For ease of distinction, the exosomes generated from the two different cultivation methods will be referred to as follows: **Pd-Exos** (obtained from placental explants cultured in complete culture medium) and **1-LPS-Pd-Exos** (referring to

exosomes obtained from placental explants co-cultured with complete culture medium containing 1μg/mL LPS). This extraction process involved a multi-step centrifugation protocol as previously described, with the final sediments being re-suspended in PBS. Following extraction, the Pd-Exos were identified via Transmission Electron Microscopy (TEM), Western Blot, and Nanoparticle Tracking Analysis (NTA). Exosomal characteristic proteins, CD63 and TSG101 (R&D Systems, Minnesota, USA) were deployed as positive controls. Calnexin (Abcam, Cambridge, UK) served as a negative control. Concurrently, Placental Alkaline Phosphatase (PLAP) (Abcam, Cambridge, UK) is a distinct protein constituent of Pd-Exos and is extensively utilized as a characteristic marker protein for these exosomes.

## Cell lines and cell culture

THP-1 cells, originally derived from the human acute monocytic leukemia cell line (Cell bank of the Chinese Academy of Science), were cultured in RPMI-1640 medium supplemented with 10% exosome-free Foetal Bovine Serum (FBS), 100U/mL penicillin, and 100μg/mL streptomycin. THP-1 cells were adjusted to 5 × 10^5 cells per well in a 24-well plate and differentiated into macrophage-like phenotype using 100 ng/mL of Phorbol 12-Myristate 13-Acetate (PMA) (Beyotime, Shanghai, China) in the RPMI-1640 complete culture medium for 48 h at 37°C under 5% $CO_2$.

Swan 71 human trophoblasts, derived from first-trimester pregnancy, were cultivated in Dulbecco's Modified Eagle Medium (Gibico, Carlsbad, CA, USA) supplemented with 10% exosome-free FBS, as well as 100U/mL penicillin and 100μg/mL streptomycin. The culture conditions were maintained at a temperature of 37°C in an incubator supplemented with 5% $CO_2$.

## Labeling and internalization of exosomes

THP-1 cells, initially grown in suspension, were co-cultured with 100 ng/mL of PMA for a period of 24 hours to stimulate differentiation into adherent macrophages. Subsequently, the isolated Pd-Exos were labeled using a PKH67 Green Fluorescent Labeling Kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's protocol. In brief, washed exosome pellets were resuspended in 250μl of Diluent C, combined with 2μl PKH67 prepared in 250μl diluent, and mixed gently for 3 minutes. An equivalent volume of 0.1% Bovine Serum Albumin (BSA) was then added to bind excess PKH67. The PKH67-labeled exosomes were recovered using ultracentrifugation and the final pellet reconstituted in PBS. Well-differentiated THP-1 cells were co-incubated with PKH67-labeled exosomes for a duration of 6 hours. Actin filaments were visualized by staining with Phalloidin (red fluorescence) (Beyotime, Shanghai, China), while the nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI) (blue fluorescence) (Abcam, Cambridge, USA). The consequent fluorescent images authenticated the internalization of the exosomes within the cells.

## THP-1 treatment with Pd-Exos

After the ultracentrifugation-based extraction of Pd-Exos, the obtained Pd-Exos from 25 ml culture supernatant were re-suspended in 250 μL of PBS. The concentration of Pd-Exos was adjusted to 1×10^9

particles/ml as determined by NTA. THP-1 cell lines were cultured in 12-well culture dishes with 5×10^5 cells per well and differentiated into macrophages by inducing with 100 ng/mL PMA. Subsequently, the cells were incubated with 100 μg/ml Pd-Exos or 1-LPS-Pd-Exos for 2h and 6h under serum-free conditions. Following the incubation period, RT-qPCR was performed to detect the gene expression of inflammatory factors. THP-1 cells treated with PBS only were used as controls at each time point for comparison and reference.

### High-throughput sequencing of exosomal miRNAs

As previously stated, two unique sets of Pd-Exos were isolated from varying sources followed by the extraction of total exosomal RNA. Four specific samples were employed to elucidate differences in miRNA expression, including four LPS induced groups and four controls. The miRNA data analysis software, ACGT101-miR (LC Sciences, Houston, Texas, USA), was employed for this process. The software's analysis workflow is as follows: (1) Removal of 3' adapters and non-essential sequences: generating clean data; (2) Length filtration: retaining animal sequences with a base length of 18-26nt; (3) Comparative and analytic assessment of multiple RNA databases: the residual sequences (excluding miRNA) were compared with mRNA, RFam and Repbase databases, followed by filtration; (4)miRNA identification: Validation of data, with miRNA identification made via comparison with precursor and genome sequences; (5)miRNA differential expression analysis; (6) Differential miRNA target gene prediction analysis. The screening threshold was set with a Log2 fold change ≧1.0 and $p < 0.05$ (Student's t test).

### Target gene prediction and Dual-Luciferase reporter assay

miR-24-1-5p's potential molecular targets were predicted using the miRDB database (https://mirdb.org/). Among the potential targets, we specifically focused on TNFAIP8, which is an integral component of the TNFAIP8/NF-κB/TNF-α pathway known for its significant involvement in inflammatory diseases [13]. A conventional method was used to construct wild-type (WT)-TNFAIP8 plasmids fused with luciferase genes and mutant (MUT) TNFAIP8 fusion genes. The logarithmic growth phase of T293 cells was harvested one day prior to transfection. Following centrifugation and suspension, the cells were inoculated into 24-well plates at a density of 1 × 10^5 cells per well. The transfections were performed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. A 50 nmol/L miR-24-1-5p mimic was co-transfected into three wells. A double-luciferase reporter activity (GeneCopoeia) system was used to compare the activity of Renilla and dual-luciferase reporters 48 hours after transfection.

### miR-24-1-5p mimics/inhibitor transfection

THP-1 cells and Swan 71 cells were separately transfected with miR-24-1-5p mimics/inhibitors (Ribobio, Guangdong, China) at a final concentration of 100 nmol/L using Lipofectamine 3000 transfection reagent when they reached 60–70% confluent in the third generation. The cells were transfected in opti-MEM (Gibco, Carlsbad, CA, USA) without FBS at 37°C and 5% $CO_2$ for 24 hours. After transfection, the

media were changed to a new culture media until harvest. Following the 24-hour transfection period, the medium was replaced with a new culture medium, and 48 hours after the initial transfection, both the cells and cell culture supernatant were harvested for further analysis.

### Western blot analysis

For Western blot analysis, cells were lysed utilizing Radioimmunoprecipitation Assay (RIPA) lysis buffer (Beyotime, Shanghai, China), complemented with Phenylmethylsulfonyl fluoride (PMSF) inhibitor (Beyotime, Shanghai, China). Following this, protein lysates were loaded and electrophoretically separated on a 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE), then electroblotted onto a 0.22 μm Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad, Hercules, California, USA). The membrane underwent blocking with 5% non-fat milk in Tris-Buffered Saline with Tween 20 (TBST) for one hour, after which it was probed with primary antibodies at 4℃ overnight. This was followed by a succession of three TBST washes, each lasting 10 minutes, prior to a one-hour incubation with secondary antibody probes. Additional three-time washing in TBST for 10 minutes each was then conducted. Proteins were visualized by employing an Enhanced Chemiluminescence (ECL) kit (Thermo Fisher, Waltham, MA, USA) and were subsequently imaged on an Image Quant LAS400 mini (GE Healthcare, Munich, Germany). Primary antibodies targeting TNF-α (Proteintech, Wuhan, China), IL-1β (Cell Signaling Technology, Boston, USA), and TNFAIP8 (Proteintech, Wuhan, China) were used. β-Actin (Cell Signaling Technology, Boston, USA) served as the standardizing loading control.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. The results are presented as mean ± standard error (SEM). Student's t-test was used for comparisons between two groups, while one-way ANOVA test was used for comparisons among three or more groups. A *p*-value less than 0.05 was considered statistically significant.

## Results

### RT-qPCR analysis of the effect of different concentrations of LPS on the expression of inflammatory genes in placental explants

In order to investigate the expression of inflammatory factors in the placental explant IAI model induced by LPS, we co-cultured the placental explants with 1 μg/ml LPS and assessed the expression of inflammatory genes at various time points (2h, 6h, 12h, 18h, and 24h) using RT-qPCR. The pro-inflammatory factors TNF-α and IL-1β, along with the anti-inflammatory factors IL-10 and IL-1Ra, were analyzed. As depicted in **Figure 1 ($A_1$-$D_1$)**, compared to the control group, LPS stimulation elicited an inflammatory response in the placental explants, resulting in a significant upregulation of pro-inflammatory factors during the early stages of stimulation (2h, 6h) [TNF-α expression increased by (10.1±2.6) fold and (14.4±5.4) fold compared to the control group, respectively, both $p < 0.05$; IL-1β expression increased by (31.0±9.9) fold and (36.0±11.4) fold compared to the control group, respectively,

both $p < 0.05$]. However, with prolonged LPS stimulation, the expression of TNF-α and IL-1β exhibited a decreasing trend (6h vs. 24h, TNF-α increased by [(14.4±5.4) fold vs. (4.0±0.5) fold], and IL-1β increased by [(36.0±11.4) fold vs. (9.9±2.4) fold], both $p < 0.05$). At 24 hours of LPS induction, there was no statistically significant difference in the expression of pro-inflammatory factors when comparing the LPS-induced group to the control group. The expression of the anti-inflammatory factor IL-10 showed no statistically significant difference among the groups as the culture time changed. However, the expression of the anti-inflammatory factor IL-1Ra demonstrated a decreasing trend with prolonged culture time (6h vs. 12h, increased by [(5.1±2.2) fold vs. (1.2±0.2) fold], $p < 0.05$). **Figure 1 ($A_2$-$D_2$)** represents the trend lines generated for the respective indices in **Figure 1 ($A_1$-$D_1$)** using the Graphpad 7.0 software.

Subsequently, we co-cultured the placental explants with 10 μg/ml LPS and examined the expression of inflammatory genes at different time points using RT-qPCR. As depicted in **Figure 1 ($E_1$-$H_1$)**, similar to the inflammatory gene expression observed with 1 μg/ml LPS stimulation, both LPS concentrations induced high expression of inflammatory genes during the early stages of stimulation, and after 18 hours of stimulation, there was a downward trend in the expression of inflammatory genes ($p < 0.05$). At 24 hours of LPS induction, the expression of inflammatory genes in the LPS-induced group did not show statistically significant differences compared to its own control group (both $p > 0.05$). **Figure 1 ($E_2$-$H_2$)** represents the trend lines generated for the respective indices in **Figure 1 ($E_1$-$H_1$)** using the Graphpad 7.0 software.

## ELISA analysis of the effect of LPS on the expression of inflammatory cytokines in the supernatant of placental explant cultures

Based on the aforementioned RT-qPCR results, we proceeded to employ a treatment of 1 μg/ml LPS on placental explants and quantified the levels of inflammatory cytokines in the culture supernatant through the ELISA method. Different time points (12h, 24h, 36h, and 48h) were set to analyze the impact of LPS on the expression of inflammatory cytokines in the placental explants (n=10). As shown in **Figure 2 ($A_1$)**, after co-culturing placental explants with 1 μg/ml LPS, the expression of the pro-inflammatory factor TNF-α exhibited a declining trend with prolonged LPS exposure [(12h vs. 48h, (444.6±287.9) vs. (227.8±126.6) pg/ml; 24h vs. 48h, (433.5±202.9) vs. (227.8±126.6) pg/ml, both $p < 0.05$)]. On the other hand, the expression of the anti-inflammatory factor IL-10 showed an increasing trend [(12h vs. 24h, (128.1±88.0) vs. (306.2±224.3) pg/ml, with statistical significance, $p = 0.031$)].**[see Figure 2 ($B_1$)]**

The ratio of pro-inflammatory to anti-inflammatory factors (TNF-α/IL-10) was used as an indicator of the pro-inflammatory direction. As shown in **Figure 2 ($C_1$)**, with increasing exposure time of placental explants to LPS, the ratio of TNF-α/IL-10 gradually decreased [(12h vs. 36h, respectively (5.3±3.9) vs. (1.8±1.2), $p < 0.05$; 12h vs. 48h, respectively (5.3±3.9) vs. (1.0±0.8), $p < 0.001$)]. As shown in **Figures 2 ($D_1$)**, the ratio of IL-10/TNF-α was used as an indicator of the anti-inflammatory direction, and with prolonged exposure time to LPS, the ratio gradually increased [(12h vs. 48h, respectively (0.5±0.2) vs.

(1.4±0.3), $p < 0.05$)]. This indicates that with prolonged exposure of placental explants to LPS, the inflammatory response shifts from a pro-inflammatory direction to an anti-inflammatory direction. **Figure 2 ($A_2$-$D_2$)** represents the trend lines generated for the respective indices in **Figure 2 ($A_1$-$D_1$)** using the Graphpad 7.0 software.

To further confirm this hypothesis, we selected another pair of pro-inflammatory factor IL-1β and anti-inflammatory factor IL-1Ra to examine their expression in placental explants co-cultured with 1 μg/ml LPS. As shown in the **Figure 2 ($E_1$),** with increasing co-culture time with LPS, there was no statistically significant difference observed in the expression of IL-1β among the different time points. However, the expression of the anti-inflammatory factor IL-1Ra showed an upward trend with prolonged culture time [(12h vs. 36h, respectively (969.3±121.0) vs. (1447.9±125.9) pg/ml; and 12h vs. 48h, respectively (969.3±121.0) vs. (1452.4±170.3) pg/ml], with statistically significant differences (both $p < 0.05$).**[(see Figure 2 ($F_1$)]**.

Using the IL-1β/IL-1Ra ratio as an indicator of the pro-inflammatory response, as shown in **Figure 2 ($G_1$)**, the inflammatory response of placental explants showed a downward trend after 36 hours of co-culture compared to 12 hours, although the difference did not reach statistical significance [(2.0±0.2) vs. (1.5±0.3), $p = 0.2785$]. Using the IL-1Ra/IL-1β ratio as an indicator of the anti-inflammatory direction, as shown in **Figure 2 ($H_1$)**, with prolonged co-culture time, placental explants exhibited a trend of inflammation inhibition, although the differences did not reach statistical significance [(12h vs. 36h, respectively (0.6±0.1) vs. (1.0±0.2), $p = 0.0774$; 24h vs. 36h, respectively (0.6±0.1) vs. (1.0 ±0.2), $p = 0.0837$]. **Figure 2 ($E_2$-$H_2$)** represents the trend lines generated for the respective indices in **Figure 2 ($E_1$-$H_1$)** using the Graphpad 7.0 software.

## ELISA analysis of the changes in inflammatory trend of placental explants cultured with normal culture medium

In order to demonstrate that placental explants cultured in a complete medium without LPS did not exhibit significant inflammation, we conducted ELISA analysis on the expression of the four inflammatory factors in the culture supernatant of placental explants from four patients at different culture time points (12h, 24h, 36h, and 48h) (n=4). As shown in the **Figure S1**, there was no increasing trend in the expression of inflammatory factors in placental explants from the same patient with prolonged culture time.

## Preliminary investigation of the molecular mechanisms underlying placental endotoxin tolerance

Based on the previous RT-qPCR analysis of gene expression and ELISA analysis of protein expression, we observed that with prolonged co-culture time with LPS, placental explants exhibited a shift in inflammatory expression from a pro-inflammatory response to an anti-inflammatory response. Specifically, during the early stages of co-culture with LPS, a significant pro-inflammatory reaction was evident, while in the later stages of culture, a distinct anti-inflammatory response was observed. The

phenomenon of endotoxin tolerance exhibited by placental tissue may play a role in maintaining an anti-inflammatory environment at the maternal-fetal interface and promoting fetal health. However, the exact mechanisms underlying the regulation of this phenomenon remain unclear.

microRNAs (miRNAs) are important cellular regulatory factors in immune responses, but there are few free circulating miRNAs in the extracellular environment, which are mainly packaged within Exosomes and act as a vehicles to transport miRNAs to target tissues or cells [9,10].

We hypothesize that prolonged exposure of placental tissue to LPS stimulation leads to the generation of "signaling molecules" that inhibit the formation of inflammatory factors, primarily immune-regulatory miRNAs. These miRNAs are encapsulated within Pd-Exos and, upon internalization by receptor cells, exert anti-inflammatory effects on target cells, thereby maintaining an anti-inflammatory environment at the maternal-fetal interface. Next, we will further investigate the possibility that this phenomenon is mediated by Pd-Exos and enclosed miRNAs, which may play a critical role in placental endotoxin tolerance at the maternal-fetal interface.

### Identification of the two groups of placenta-derived exosomes (Pd-Exos) through transmission electron microscopy (TEM), western blot (WB), and nanoparticle tracking analysis (NTA) techniques

The LPS-induced group and its respective control group were each subjected to the extraction of Pd-Exos from the culture supernatant of four pairs of samples. Following purification, the exosomes were stained with 2% uranyl acetate for observation under transmission electron microscopy (TEM). Both groups displayed typical exosome structures characterized by a double-layered "saucer-shaped" membrane **[Figure 3 (A-B)]**. Western Blot analysis revealed that the extracted Pd-Exos expressed specific markers of classic exosomes, including the tetraspanin protein CD63 and the endosomal transport protein marker TSG101, while lacking expression of the endoplasmic reticulum-specific molecule calnexin (used as a negative control). Additionally, the placenta-specific marker placental alkaline phosphatase (PLAP) showed positive expression, confirming the origin of these exosomes from placental sources **[Figure 3 (C)]**. Nanoparticle tracking analysis (NTA) revealed that the diameter of exosomes in the control group was (137.6±9.8) nm, and in the LPS-induced group was (135.9±5.9) nm, both falling within the characteristic diameter range of exosomes (30~150 nm). Collectively, this evidence indicates successful enrichment of Pd-Exos through ultracentrifugation **[Figure 3 (D-E)]**.

### Interaction between Pd-Exos and inflammatory cells at the maternal-fetal interface

Human monocytic leukemia cell line THP-1 was induced with 100 ng/ml PMA for 24 hours to differentiate into adherent macrophages, which served as the cellular model for inflammatory cells at the maternal-fetal interface. We used this model to investigate the impact of Pd-Exos on the expression of inflammatory genes in maternal-fetal interface macrophages.

### PKH67-labelled exosomes internalize by THP-1 cells

THP-1 cells were selected and cultured in complete medium containing 100 ng/ml PMA for 24 hours to induce their differentiation into macrophages. PKH67-labeled Pd-Exos (green fluorescent) were added to the culture and co-cultured for 6 hours. Immunofluorescence analysis confirmed the internalization of Pd-Exos by THP-1 cells. As shown in **Figure 4 (A-D),** confocal microscopy imaging demonstrated the internalization of PKH67-labeled green fluorescent Pd-Exos by THP-1 cells.

### Impact of Pd-Exos treatment on the expression of inflammatory genes in THP-1 cells

### (1) THP-1 treated with Pd-Exos

RT-qPCR analysis was performed to assess the expression of inflammatory genes in THP-1 cells after co-culturing with Pd-Exos for 2 hours and 6 hours. The pro-inflammatory factors TNF-α and IL-10, as well as the anti-inflammatory factors IL-1β and IL-1Ra, were investigated. Comparing with the control group of THP-1 cells, after 2 hours of co-culturing with Pd-Exos, there was a significant upregulation of TNF-α mRNA expression (1.95±0.23-fold). However, there were no statistically significant differences in the expression of IL-10, IL-1β, and IL-1Ra. After 6 hours of co-culturing, Pd-Exos promoted the expression of IL-1β (2.93±0.89-fold, $p$ <0.01) and significantly upregulated the expression of the anti-inflammatory factors IL-10 (1.85±0.20-fold) and IL-1Ra (6.02±3.31-fold). The expression of TNF-α showed no statistically significant difference**[see Figure 5 ($A_1$-$D_1$)]**.The TNF-α/IL-10 ratio, as an indicator of pro-inflammatory direction, showed a decreasing trend after 2 hours and 6 hours of co-culturing Pd-Exos with THP-1 cells, from (1.73±0.18) to (1.03±0.29), although the difference did not reach statistical significance ($p$ =0.066) **[Figure 5 ($E_1$)]**. On the other hand, the IL-1β/IL-1Ra ratio, as an indicator of pro-inflammatory direction, exhibited a significant decrease after 2 hours and 6 hours of co-culturing, from (1.57±0.19) to (0.70±0.10) ($p$ <0.01)**[Figure 5 ($F_1$)]**. Our results indicate that Pd-Exos can mildly promote inflammation in THP-1 cells after 2 hours of interaction, but this pro-inflammatory effect gradually weakens with prolonged co-culturing up to 6 hours.

1. **THP-1 treated with 1-LPS-Pd-Exos**

Using the same methodology as described above, we examined the impact of 1-LPS-Pd-Exos on the expression of inflammatory genes in THP-1 cells. Comparing with the control group, after 2 hours of co-culturing 1-LPS-Pd-Exos with THP-1 cells, there was a significant increase in TNF-α (6.00±1.26-fold), IL-10 (1.52±0.16-fold), and IL-1β (1.63±0.23-fold) mRNA expression. However, the expression of the anti-inflammatory factor IL-1Ra was downregulated (0.57±0.09-fold), and all differences were statistically significant ($p$ <0.05). After 6 hours of co-culturing, 1-LPS-Pd-Exos continued to promote the expression of TNF-α (4.63±1.31-fold), IL-10 (2.90±0.23-fold), and IL-1β (6.18±1.51-fold). Interestingly, the expression of IL-1Ra changed (6.65±1.46-fold) from inhibition to significant promotion **[see Figure 5 ($A_2$-$D_2$)]**. when considering the TNF-α/IL-10 ratio as an indicator of pro-inflammatory direction, 1-LPS-Pd-Exos and THP-1 co-culturing for 2 hours and 6 hours resulted in a decreasing trend in this ratio, from (3.80±0.47) to (1.63±0.44), with statistically significant differences ($p$ <0.01) **[Figure 5 ($E_2$)]**. Similarly, when considering the IL-1β/IL-1Ra ratio as an indicator of pro-inflammatory direction, 1-LPS-Pd-Exos and THP-1 co-

culturing for 2 hours and 6 hours showed a decreasing trend in this ratio, from (3.03±0.32) to (1.02±0.26), with statistically significant differences ($p$ <0.001) **[Figure 5 ($F_2$)]**. These results indicate that 1-LPS-Pd-Exos can induce a pro-inflammatory response in THP-1 cells after 2 hours of interaction, and this pro-inflammatory effect gradually diminishes with prolonged co-culturing up to 6 hours.

## Screening of differentially expressed placenta-derived exosomal miRNAs related to placental endotoxin tolerance

To investigate the expression profiles of differentially expressed miRNAs associated with placental endotoxin tolerance (ET) in Pd-Exos, we selected 4 pairs of Pd-Exos samples for analysis. (Note: Each pair of samples was derived from placental explant cultures of the same pregnant woman under two different culture conditions, namely Pd-Exos, n=4; and 1-LPS-Pd-Exos, n=4). Total RNA was extracted and subjected to miRNA high-throughput sequencing and bioinformatics analysis. The internationally recognized algorithm DESeq2 was employed for differential screening, with the screening criteria set at $Log_2FC > 1$ and $p < 0.05$. A total of 6 differentially expressed miRNAs were identified. The differentially expressed miRNAs, fold change, and expression levels are shown **in Table 1** (Note: FC=fold change).

The miRNA sequencing results revealed 1025 differentially expressed miRNAs in 4 pairs of Pd-Exos samples. Among them, 21 miRNAs were consistently identified in all pairs **(Figure 6-A)**. Hierarchical clustering analysis was performed for the 1025 differentially expressed miRNAs in the 4 pairs of Pd-Exos samples **(Figure 6-B)**. Based on the fold change and expression levels, we selected miR-24-1-5p, which showed the most significant differential expression, for further investigation and subsequent functional validation **(Figure 6-C).** To elucidate the functions of miRNA-regulated genes in different biological processes, we conducted GO enrichment analysis on the mRNA targets potentially influenced by miRNAs in the Pd-Exos samples. The results indicated that the enriched miRNAs primarily functioned in processes related to protein binding, cytoplasm, nucleoplasm, and cytosol, among others **(Figure 6-D)**.

## Prediction of the target genes of miR-24-1-5p

Based on the previous miRNA high-throughput sequencing results, we selected miR-24-1-5p with the highest differential expression for further functional validation. Using the miRDB database, we predicted potential target genes of miR-24-1-5p and found TNFAIP8 (TNF alpha induced protein 8) as a potential target, which is expressed in human placental tissues and located on chromosome 5q23.1. Combining with the literature, we further hypothesize that placental-derived exosomal miR-24-1-5p may inhibit the transcription of TNFAIP8 mRNA, thereby suppressing the activation of the NF-κB inflammatory pathway and reducing the secretion of inflammatory factors TNF-α and IL-1β [13]. (The proposed mechanism of miR-24-1-5p involvement in placental Enodtoxin Tolerance is depicted in **Figure 7**.)

## miR-24-1-5p is highly expressed in 1-LPS-Pd-Exos released from LPS-induced placental explants compared to Pd-Exos from the control group

In order to validate the accuracy of the sequencing results, we additionally selected 5 samples of term, cesarean delivery placental explant tissues from pregnant women without pregnancy complications. Using the same placental explant culture method as described earlier, we cultured the explants with either complete medium or complete medium containing 1μg/ml LPS. After 18 hours of culture, a total of 10 exosome samples were extracted from the culture supernatant of placental explants (including Pd-Exos, n=5, and 1-LPS-Pd-Exos, n=5). RT-qPCR analysis showed that the expression of miR-24-1-5p in 1-LPS-Pd-Exos released from LPS-induced placental explants was (2.74±0.71) fold higher than the control group, and this difference was statistically significant ($p < 0.001$), which is consistent with the sequencing results.**Figure S2**

### miR-24-1-5p directly targets the 3'UTR region of TNFAIP8

Using the dual luciferase reporter vector pmiR-RB-Report$^{TM}$, which simultaneously expresses firefly luciferase and Renilla luciferase activities, we constructed the wild-type TNFAIP8 gene 3'Untranslated Region (UTR) recombinant plasmid h-TNFAIP8-WT, containing the binding site of miR-24-1-5p. Additionally, we constructed the 3'UTR mutant plasmid h-TNFAIP8-MUT, with 7 nucleotide mutations at the miR-24-1-5p binding site. Using luciferase activity assay, we observed the effect of miR-24-1-5p on the firefly luciferase activity of h-TNFAIP8-UTR plasmids. The results showed that co-transfection of miR-24-1-5p mimic with h-TNFAIP8-WT significantly downregulated reporter vector luciferase activity (61% downregulation) compared to the miR-24-1-5p NC group, with statistical significance ($p$ =0.0002). In contrast, no significant changes in luciferase activity were observed in the co-transfection group with h-TNFAIP8-MUT ($p$ =0.18), suggesting that miR-24-1-5p likely regulates gene expression through its action on the 3'UTR region of the TNFAIP8 gene. **[Figure 8 (A-B)]**

### Effect of miR-24-1-5p overexpression in recipient cells on target genes and downstream inflammatory cytokine expression

### (1)Transfection of miR-24-1-5p into THP-1 cell line (Paracrine mechanism)

### RT-qPCR Analysis

To investigate the impact of miR-24-1-5p on inflammation in the maternal-fetal interface, we transfected well-differentiated THP-1 cells with 100 nmol/L of miR-24-1-5p mimic, mimicking the effect of miR-24-1-5p on maternal-fetal interface inflammatory cells. Our results showed that, compared to the negative control (NC) group, transfection with miR-24-1-5p mimic led to a downregulation of the target gene TNFAIP8 in THP-1 cells by approximately 83% ± 2% (corresponding to a decrease of approximately 17%). Furthermore, the expression of downstream inflammatory cytokines TNF-α and IL-1β was downregulated to approximately 54% ± 8% and 47% ± 7%, respectively. All observed differences were statistically significant. **[Figure 9 ($A_1$)]**

### Western blot Analysis

After transfecting THP-1 cell line with miR-24-1-5p mimic/inhibitor for 72 hours, cells were collected, and total proteins were extracted. Western Blot analysis was conducted to assess the protein levels of the target gene TNFAIP8, as well as the expression of downstream inflammatory factors TNF-α and IL-1β. The results demonstrated that compared to the negative control group (NC), transfection with miR-24-1-5p mimic for 72 hours resulted in a reduction in the protein expression of the target gene TNFAIP8, as well as a downward trend in the expression of IL-1β precursor and TNF-α, all with statistical significance ($p < 0.05$). Conversely, when compared to the negative control group, transfection with miR-24-1-5p inhibitor led to an increase in the expression of IL-1β precursor and TNF-α, both showing statistically significant differences ($p < 0.05$) (relative grayscale values are indicated in the bar chart, represented as mean ± standard error).**[Figure 9 ($B_1$)]**

### ELISA

After transfecting THP-1 cell line with miR-24-1-5p for 72 hours, cell culture supernatants were collected, and ELISA was performed to measure the expression levels of inflammatory factors TNF-α and IL-1β. The results showed that compared to the negative control group (mimic NC), transfection with miR-24-1-5p mimic for 72 hours led to a decrease in the expression of TNF-α and IL-1β in the cell culture supernatant, with TNF-α levels being [(373.2±52.3) pg/mL vs. (740.9±41.9) pg/mL] and IL-1β levels being [(85.3±24.5) pg/mL vs. (33.8±7.3) pg/mL], both with statistical significance ($p < 0.05$). Conversely, when compared to the negative control (Inhibitor NC), transfection with miR-24-1-5p inhibitor for 72 hours resulted in an upregulation of TNF-α and IL-1β expression in the cell culture supernatant, with TNF-α levels being [(116.5±17.4) pg/mL vs. (66.1±3.3) pg/mL] and IL-1β levels being [(25.9±5.9) pg/mL vs. (15.9±1.6) pg/mL], both with statistical significance ($p < 0.05$). **[Figure 9 ($C_1$)]**

## (2)Transfection of miR-24-1-5p into Swan 71 cell line (Autocrine mechanism)

### RT-qPCR Analysis

Pd-Exos and their cargo of miRNAs can exert their effects through both paracrine mechanisms on maternal-fetal interface cells (such as macrophages) and autocrine mechanisms on placental tissue itself, including trophoblast cells [14].Subsequently, Pd-Exos utilize homologous surface markers to interact with target tissues/cells and release their cargo, thereby influencing various biological mechanisms, including protein biosynthesis and/or post-transcriptional regulation. To further investigate the inflammatory regulatory role of miR-24-1-5p in the placental tissue itself, we used 100nmol/L of miR-24-1-5p mimic to transfect Swan71 trophoblast cell line, simulating the interaction between Pd-Exos and trophoblast cells and their cargo. The results showed that compared to the NC group, after transfection with miR-24-1-5p mimic, the expression of the target gene TNFAIP8 in Swan71 cells was downregulated to (50.7% ± 8.3%, i.e., a decrease of 49.3%), and the downstream inflammatory factors TNF-α and IL-1β were downregulated to approximately (57.0% ± 5.2%) and (58.7% ± 10.8%), respectively, with all differences being statistically significant (all $p < 0.05$). **[Figure 9 ($A_2$)]**

### Western blot analysis

After transfecting Swan71 cells with miR-24-1-5p mimic for 72 hours, cells were collected, and total protein was extracted. Western blot analysis was performed to assess the protein levels of the target gene TNFAIP8, as well as the expression of downstream inflammatory factors TNF-α and IL-1β. The results showed that compared to the negative control group (mimic NC), transfection with miR-24-1-5p mimic for 72 hours led to a decrease in the protein expression of the target gene TNFAIP8 and TNF-α, with statistical significance (both $p < 0.05$). Additionally, the expression of IL-1β precursor showed a decreasing trend, although the difference did not reach statistical significance. On the other hand, compared to the negative control group (Inhibitor NC), transfection with miR-24-1-5p Inhibitor resulted in an upregulation of TNFAIP8 and TNF-α expression, with statistical significance (both $p < 0.05$). Similarly, the expression of IL-1β precursor showed an increasing trend, but the difference did not reach statistical significance. (Relative grayscale values are indicated in the bar charts, represented as mean ± standard error).**[Figure 9 ($B_2$)]**

### ELISA

After transfecting with miR-24-1-5p mimic for 72 hours, the cell culture supernatant was collected, and the expression of inflammatory factors TNF-α and IL-1β was detected using the ELISA technique. The results showed that compared to the negative control group (mimic NC), transfection with miR-24-1-5p mimic for 72 hours led to a decrease in the expression of TNF-α and IL-1β, with values of TNF-α [(14.5±0.15) pg/mL vs. (21.0±0.87) pg/mL] and IL-1β [(11.4±0.3) pg/mL vs. (13.6±0.8) pg/mL], respectively (both $p < 0.05$). Compared to the negative control, transfection with miR-24-1-5p Inhibitor for 72 hours resulted in an upregulation of TNF-α expression, with a value of TNF-α [(13.9±0.3) pg/mL vs. (16.6±1.3) pg/mL], while the expression of IL-1β showed an increasing trend, but the difference did not reach statistical significance, with values of [(8.5±0.8) pg/mL vs. (9.4±0.5) pg/mL], $p = 0.135$. **[Figure 9 ($C_2$)]**

# Disscussion

Intra-amniotic infection (IAI), also known as chorioamnionitis, refers to the collective term for infections of the amniotic fluid, fetal membranes, placenta, or fetus caused by invading pathogens. It can lead to severe maternal and fetal complications.[3] Currently, the specific pathogenesis and the role of the placenta in this process remain unclear. Establishing a reproducible and stable animal model of IAI is of great significance for in-depth research into its pathogenesis, occurrence, and development, as well as exploring drug treatments or early interventions.

Currently, animal models for studying IAI mainly consist of rodents, sheep, pigs, and other animals [15-17]. However, due to the highly diverse nature of placental species, evidence from animal models derived from human placental diseases is limited [18]. Consequently, there is growing attention towards using human-derived placental explants co-cultured with LPS to establish a simple placental model and investigate the role of placental tissue in regulating inflammation at the maternal-fetal interface [19,20]. Recently, researchers have utilized full-term placental explants infected with SARS-CoV-2019 to study the inflammatory response at the maternal-fetal interface caused by the virus [21]. There are relevant

methods available for isolating and culturing placental explants from full-term placentas [22,23]. The placental explant system partially maintains the three-dimensional structure of the tissue, allowing for interactions between cells and better representing the in vivo situation than isolated primary trophoblast cells or cell lines [24]. Therefore, to investigate the biological functions of human placental tissue at the maternal-fetal interface during the occurrence and development of intrauterine infection, we employed an ex vivo model based on cultured human placental explants and evaluated the placental tissue's response to LPS stimulation.

Our results demonstrate that in the current IAI model, LPS can induce an inflammatory response in placental explants. During the early co-culture period, the placental explants exhibited a significant pro-inflammatory response. However, during the later co-culture period, the pro-inflammatory response gradually diminished, while the anti-inflammatory response increased. This indicates that with prolonged co-culture time with LPS, the placental explants' inflammatory expression shifts from a pro-inflammatory to an anti-inflammatory direction. Boles et al. established a murine placental explant infection model using placental tissues from preterm (e16) and near-term (e20) pregnant mice co-cultured with different concentrations of LPS (0, 1, 10μg/ml) [9]. Their research revealed that compared to near-term (e20) placental explants, LPS induced higher expression of IL-6 and TNF-α in preterm placental explants, suggesting that preterm fetal placenta may exhibit a greater cytokine response to LPS stimulation. Kim et al. selected human mid-term placental tissues, isolated placental explants, and subjected them to repeated co-culture with 100ng/ml LPS [20]. They analyzed the culture supernatant every 24 hours and replaced it with fresh LPS-containing medium for repetitive stimulation. Their findings demonstrated that mid-term human placental tissues, repeatedly exposed to LPS stimulation, exhibited inducible tolerance to endotoxin stimulation. This repetitive exposure to LPS led to a significant decrease in pro-inflammatory factors and an increase in anti-inflammatory factor secretion. Our research results are consistent with these findings. Furthermore, their study also discovered that the viability of the explants only decreased by 19% during the 1 to 4 days of culture, indicating that the explants maintained good vitality [20].

Inflammation is a complex pathological and physiological state primarily elicited by the response of innate immune cells to inflammation or tissue damage. This response is triggered through pattern recognition receptors (PRRs) expressed on their surfaces, leading to a robust and necessary inflammatory reaction. However, this process requires strict control. If inflammation is not properly regulated, it can result in extensive tissue damage and manifestation of pathological conditions [25]. One of the physiological adaptations the body uses to regulate excessive inflammatory response is called Endotoxin Tolerance [26]. This phenomenon occurs when cells or the organism are exposed to an initial low dose of endotoxin, entering a transient state of unresponsiveness and failing to react to further endotoxin stimulation. In other words, they exhibit a form of "tolerance" to endotoxin stimulation [27]. However, this tolerance is not "immunoparalysis" but rather an activation of an alternative mechanism yet to be fully described [25]. Endotoxin Tolerance is a protective phenomenon and serves as a defense mechanism by the body against inflammation. It can ameliorate symptoms such as fever, hypoxia, and shock. Dysregulation of endotoxin tolerance may lead to conditions like sepsis, cystic fibrosis (CF), acute coronary syndrome (ACS), trauma, and pancreatitis [25]. Recent research has also indicated that the

failure of placental tolerance to repetitive endotoxin stimulation might be a contributing mechanism to the occurrence of inflammatory preterm birth at the maternal-fetal interface [28]. However, the exact mechanisms underlying placental endotoxin tolerance remain unclear.

miRNA is an important regulatory factor in immune responses [9]. In recent years, research has also identified miRNAs, such as miR-146a, miR-155, and miR-98, as being associated with Endotoxin Tolerance [29-31]. Existing evidence indicates that miRNAs play crucial roles in maintaining normal physiological pregnancy and are involved in immune regulation at the maternal-fetal interface [32-36]. Moreover, miRNAs are not only freely circulating in extracellular fluids but are also packaged within extracellular vesicles (EVs) generated by various cells and tissues [14]. Placental tissue, in particular, is known to produce abundant and transient Pd-Exos [14,28]. These exosomes, containing miRNAs, are secreted into the extracellular environment through processes such as endocytosis, fusion, and exocytosis. The miRNAs within these exosomes mainly bind to target gene mRNAs in recipient cells, thereby inhibiting their stability and protein synthesis. These miRNAs are selectively secreted to mediate cell-to-cell signaling and intercellular communication [28].

Therefore, this study aimed to explore the molecular mechanism of placental endotoxin tolerance from the perspective of Pd-Exos and their encapsulated miRNAs. For the first time, our research revealed that prolonged exposure to LPS stimulation led to higher expression of miR-24-1-5p in exosomes derived from placental explants. These exosomes were internalized by recipient cells via paracrine and autocrine pathways. Subsequently, miR-24-1-5p suppressed the expression of TNFAIP8 mRNA in target cells, resulting in the inhibition of downstream inflammatory factors, such as TNF-α and IL-1β. This discovery unveils a novel mechanism involving miR-24-1-5p in placental endotoxin tolerance.

Tumor necrosis factor-alpha-induced protein 8 (TNFAIP8) was the first identified member of the TIPE (Tumor necrosis factor-alpha-induced protein 8-like) family, which includes four members: TNFAIP8, TIPE1 (TNFAIP8L1), TIPE2 (TNFAIP8L2), and TIPE3 (TNFAIP8L3). Increasing evidence suggests that TNFAIP8 plays a crucial role in the regulation of various tumors and inflammatory diseases [37-39]. Recent studies have also revealed its involvement in the regulation of inflammatory diseases, particularly in the context of bacterial infections and inflammatory disorders [39]. However, the functional research on miR-24-1-5p is still in the exploratory stage. Stikbakke et al. found that high expression of miR-24-1-5p is associated with an increased risk of prostate cancer recurrence after radical prostatectomy, indicating its potential diagnostic and therapeutic value in prostate cancer cases [40]. Overexpression of miR-24-1-5p in human colorectal cancer cells significantly inhibits β-catenin expression and reduces cell proliferation, migration, and survival [41]. In a C57BL/6 mouse animal model, maternal consumption of a high-protein diet upregulates miR-24-1-5p expression, leading to downregulation of the target gene SMAD-5 mRNA and inhibiting bone mass in offspring mice [42]. However, there are currently no relevant reports on the role of miR-24-1-5p in placental inflammatory diseases.

Our study possesses several notable strengths. Firstly, we employed the placental explant system, which partially preserves the three-dimensional villous structure and facilitates cell interactions, in contrast to

using isolated trophoblast cells or cell lines [24]. Prior research has validated that placental explant culture maintains the intact structure of the syncytiotrophoblast and cytotrophoblast layers in the villous regions [20]. By selectively obtaining healthy, full-term placental explants from elective cesarean sections, we acquired suitable samples to investigate the immune role of placental tissue during the development of IAI. Secondly, we successfully established an IAI model by co-culturing placental explants with LPS and continuously monitoring the expression of inflammatory factors at different time points. This approach enabled us to control for the specificity of the causative agent of IAI while studying the immune response of placental tissue at various infection stages. Thirdly, through differential miRNA sequencing of Pd-Exos, we identified a novel miRNA, miR-24-1-5p, which may be implicated in placental endotoxin tolerance. We conducted preliminary functional research on this miRNA.

However, our study still has several limitations. Firstly, we employed LPS to co-culture with placental explants as a modeling approach to investigate the immune regulatory role of the placenta during the development of IAI. As a result, our findings can only explain the immune response to IAI caused by the most common Gram-negative bacterium, Escherichia coli. Different types of IAI may involve distinct mechanisms that were not covered in this study. Secondly, we identified a novel miRNA, miR-24-1-5p, which might be involved in the placental endotoxin tolerance mechanism. We validated this finding through cell experiments. However, the functional significance of miR-24-1-5p in the complex microenvironment of the organism requires further confirmation, possibly through animal models or other approaches.

In summary, we established an in vitro Intra-Amniotic Infection (IAI) model by co-culturing LPS with placental explants and conducted inflammation trend analysis, which demonstrated that LPS induced inflammation in placental explants, providing a straightforward approach for studying IAI. In the early stages of the LPS-induced IAI model, the placental tissue exhibits a notable pro-inflammatory response. However, prolonged exposure to LPS leads to the development of placental endotoxin tolerance. Prolonged LPS stimulation of placental explants results in the production of Pd-Exos containing elevated levels of miR-24-1-5p. Upon internalization by recipient cells, miR-24-1-5p suppresses the expression of the target gene TNFAIP8 in recipient cells, thereby inhibiting the expression of inflammatory factors TNF-α and IL-1β. This mechanism is involved in placental endotoxin tolerance. Further research may reveal therapeutic targets for managing obstetric inflammatory-related diseases.

# Abbreviations

| | |
|---|---|
| IAI | Intra-amniotic infection |
| LPS | Lipopolysaccharide |
| Pd-Exos | Placenta-derived exosomes |
| TNFAIP8 | Tumor necrosis factor alpha-induced protein 8 |

| | |
|---|---|
| NTA | Nanoparticle tracking analysis |
| TEM | Transmission electron microscopy |
| ELISA | Enzyme-linked immunosorbent assay |
| RT-qPCR | Real-time quantitative polymerase chain reaction |

# Declarations


### Acknowledgments

This work was supported by the National Key Research and Development Program of China (Grant No. *2021YFC2700700*). We acknowledge the valuable assistance provided by Menglin Zhou, Xinning Chen, Yinluan Ouyang and Long Bai in the preparation of this manuscript.


### Author Contributions

Yongqing Zhang and Danqing Chen conceived the study. Yongqing Zhang performed data analysis and drafted the initial manuscript. Lejun Li and Luping Chen participated in the analysis of RT-qPCR and miRNA-seq analyses. Lejun Li and Tiantian Fu contributed to gene function experiments. Luping Chen, Guohui Yan, and Tiantian Fu conducted the ELISA analysis, while Luping Chen and Yongqing Zhang were responsible for the collection, cultivation, and dosing time exploration of placental explants. Yongqing Zhang and Lejun Li were involved in the final data analysis, interpretation, and manuscript writing. Meihua Sui and Danqing Chen revised the manuscript. All authors have read and approved the publication of this manuscript.

### Declaration of interests

The authors declare no competing interests.

### Data availability statement

The original contributions presented in this study are included in the article/supplementary material, and further inquiries can be directed to the corresponding authors.

# References


1. McMaster K, Sanchez-Ramos L, Kaunitz AM. Evaluation of a transcervical foleycatheter as a source of infection: a systematic review and meta-analysis. Obstet Gynecol. 2015;126:539-51.
2. Gommers JSM, Diederen M, Wilkinson C, Turnbull D, Mol BW. Risk of maternal, fetal and neonatal complications associated with the use of the transcervical balloon catheter in induction of labour: a systematic review. Eur J Obstet Gynecol Reprod Biol. 2017;218:73-84.

3. Committee on Obstetric Practice: Committee opinion no. 712: intrapartum management of intraamniotic infection. Obstet Gynecol. 2017;130:e95–e101.
4. Zhang Y, Yu Y, Chen L, Zhao W, Chu K, Han X. Risk Factors of Intra-Amniotic Infection Related to Induction with Single-Balloon Catheter: A Case-Control Study. Gynecol Obstet Invest. 2019;84(2):183-9.
5. Zhang Y, Chen L, Yan G, Zhou M, Chen Z, Liang Z, et al. Comparison of the effectiveness and pregnancy outcomes of labor induction with dinoprostone or single-balloon catheter in term nulliparous women with borderline oligohydramnios. Chin Med J (Engl). 2022;135(6):681-90.
6. Fan SR, Liu P, Yan S, Peng J, Liu X. Diagnosis and Management of Intraamniotic Infection. Maternal-Fetal Medicine. 2020;2:223-30.
7. Erlebacher A. Immunology of the maternal-fetal interface. Annu Rev Immunol. 2013;31:387-411.
8. Fu X, Li Y, Zhang Z, et al. Emerging role of miRNAs, lncRNAs, and circRNAs in pregnancy-associated diseases. Chin Med J (Engl). 2023;136(11): 1300-10.
9. Pua HH, Steiner DF, Patel S, Gonzalez JR, Ortiz-Carpena JF, Kageyama R, et al. MicroRNAs 24 and 27 Suppress Allergic Inflammation and Target a Network of Regulators of T Helper 2 Cell-Associated Cytokine Production. Immunity. 2016;19;44(4):821-32.
10. Hadley EE, Sheller-Miller S, Saade G, Salomon C, Mesiano S, Taylor RN, et al. Amnion epithelial cell-derived exosomes induce inflammatory changes in uterine cells. Am J Obstet Gynecol. 2018;219(5):478.e1-478.e21.
11. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. Physiol Rev. 2016;96:1297-325.
12. Mathieu M, Martin-Jaular L, Lavieu G, Thery C: Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat Cell Biol. 2019;21:9-17.
13. Hua J, Zhuang G, Qi Z. Current research status of TNFAIP8 in tumours and other inflammatory conditions (Review). Int J Oncol. 2021;59(1):1-12.
14. Kamity R, Sharma S, Hanna N. MicroRNA-Mediated Control of Inflammation and Tolerance in Pregnancy. Front Immunol. 2019;10:718.
15. Greenberg JM, Romero R, Winters AD, Galaz J, Garcia-Flores V, Arenas-Hernandez M, et al. Microbiota of the Pregnant Mouse: Characterization of the Bacterial Communities in the Oral Cavity, Lung, Intestine, and Vagina through Culture and DNA Sequencing. Microbiol Spectr. 2022;10(4):e0128622.
16. Kramer BW, Glaser K. Ureaplasma-Driven Neonatal Neuroinflammation: Novel Insights from an Ovine Model. Cell Mol Neurobiol. 2023;43(2):785-95.
17. Splichal I, Splichalova A. High Mobility Group Box 1 in Pig Amniotic Membrane Experimentally Infected with E. coli O55. Biomolecules. 2021; 11(8):1146.
18. Shamir E.R., Ewald A.J. Three-dimensional organotypic culture: experimental models of mammalian biology and disease. Nat. Rev. Mol. Cell Biol. 2014;15:647–64.

19. ccBoles JL, Ross MG, Beloosesky R, Desai M, Belkacemi L. Placental-mediated increased cytokine response to lipopolysaccharides: a potential mechanism for enhanced inflammation susceptibility of the preterm fetus. J Inflamm Res. 2012;5:67-75.
20. Kim ML, Maloney C, Klimova N, Gurzenda E, Lin X, Arita Y, et al. Repeated lipopolysaccharide exposure leads to placental endotoxin tolerance. Am J Reprod Immunol. 2019;81(2):e13080.
21. Zumkehr B, Oliveira Esteves BI, Bracher L, Wotzkow C, Blank F, Thiel V, et al. SARS-CoV-2 can infect and propagate in human placenta explants. Cell Rep Med. 2021;2(12):100456.
22. Colson A, Depoix CL, Hubinont C, Debiève F. Isolation of Primary Cytotrophoblasts From Human Placenta at Term. Bio Protoc. 2021;11(19):e4185.
23. Tong M, Chamley LW. Isolation and Characterization of Extracellular Vesicles from Ex Vivo Cultured Human Placental Explants. Methods Mol Biol. 2018; 1710:117-29.
24. Alhareth K, Valero L, Mohamed KE, Fliedel L, Roques C, Gil S, et al. Qualitative and quantitative analysis of the uptake of lipoplexes by villous placenta explants. Int J Pharm. 2019;567:118479.
25. López-Collazo E, del Fresno C. Pathophysiology of endotoxin tolerance: mechanisms and clinical consequences. Crit Care. 2013;17(6):242.
26. Cavaillon JM, Adib-Conquy M. Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Crit Care. 2006;10(5):233.
27. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 2009;30(10):475-87.
28. Tiozzo C, Bustoros M, Lin X, Manzano De Mejia C, Gurzenda E, Chavez M, et al. Placental extracellular vesicles-associated microRNA-519c mediates endotoxin adaptation in pregnancy. Am J Obstet Gynecol. 2021;225(6):681. e1-681.e20.
29. Nahid MA, Pauley KM, Satoh M, Chan EK. miR-146a is critical for endotoxin-induced tolerance: implication in innate immunity. J Biol Chem. 2009;284(50): 34590-99.
30. Liu Y, Wan X, Yuan Y, Huang J, Jiang Y, Zhao K, et al. Opposite effects of miR-155 in the initial and later stages of lipopolysaccharide (LPS)-induced inflammatory response. J Zhejiang Univ Sci B. 2021;22(7):590-98.
31. Liu Y, Chen Q, Song Y, Lai L, Wang J, Yu H, Cao X, Wang Q. MicroRNA-98 negatively regulates IL-10 production and endotoxin tolerance in macrophages after LPS stimulation. FEBS Lett. 2011;585(12):1963-8.
32. Tong M, Abrahams VM, Chamley LW. Immunological effects of placental extracellular vesicles. Immunol Cell Biol. 2018;96:714-22.
33. Salomon C, Scholz-Romero K, Sarker S, Sweeney E, Kobayashi M, Correa P, et al. Gestational Diabetes Mellitus Is Associated With Changes in the Concentration and Bioactivity of Placenta-Derived Exosomes in Maternal Circulation Across Gestation. Diabetes. 2016;65(3):598-609.
34. Elfeky O, Longo S, Lai A, Rice GE, Salomon C. Influence of maternal BMI on the exosomal profile during gestation and their role on maternal systemic inflammation. Placenta. 2017;50:60-9.

35. Holder B, Jones T, Sancho Shimizu V, Rice TF, Donaldson B, Bouqueau M, et al. Macrophage Exosomes Induce Placental Inflammatory Cytokines: A Novel Mode of Maternal-Placental Messaging. Traffic. 2016;17(2):168-78.
36. Holder BS, Tower CL, Jones CJ, Aplin JD, Abrahams VM. Heightened pro-inflammatory effect of preeclamptic placental microvesicles on peripheral blood immune cells in humans. Biol Reprod. 2012;86(4):103.
37. Lou Y, Liu S. The TIPE (TNFAIP8) family in inflammation, immunity, and cancer. Mol Immunol. 2011;49(1-2):4-7.
38. Sun H, Lou Y, Porturas T, Morrissey S, Luo G, Qi J, Ruan Q, Shi S, Chen YH. Exacerbated experimental colitis in TNFAIP8-deficient mice. J Immunol. 2015;194(12):5736-42.
39. Porturas TP, Sun H, Buchlis G, Lou Y, Liang X, Cathopoulis T, et al. Crucial roles of TNFAIP8 protein in regulating apoptosis and Listeria infection. J Immunol. 2015;194(12):5743-50.
40. Stikbakke E, Wilsgaard T, Haugnes HS, Pedersen MI, Knutsen T, Støyten M, et al. Expression of miR-24-1-5p in Tumor Tissue Influences Prostate Cancer Recurrence: The PROCA-life Study. Cancers (Basel). 2022;14(5):1142.
41. Zhang H, Guo J, Mao L, Li Q, Guo M, Mu T, et al. Up-regulation of miR-24-1-5p is involved in the chemoprevention of colorectal cancer by black raspberry anthocyanins. Br J Nutr. 2019;122(5):518-26.
42. Ellur G, Sukhdeo SV, Khan MT, Sharan K. Maternal high protein-diet programs impairment of offspring's bone mass through miR-24-1-5p mediated targeting of SMAD5 in osteoblasts. Cell Mol Life Sci. 2021;78(4):1729-44.

# Table 1

**Table 1 Screening results of differentially expressed placenta-derived miRNAs associated with placental endotoxin tolerance**

| Differentially miRNAs | Fold Change LPS/control | Expressed in LPS group | Exo_Con (Mean) | Exo_LPS (Mean | *p* value |
|---|---|---|---|---|---|
| hsa-miR-24-1-5p | 7.41 | up | 1.3 | 10 | 0.00363 |
| hsa-miR-7977 | 2.47 | up | 15 | 36 | 0.0220 |
| hsa-miR-4521 | 6.76 | up | 1.0 | 7 | 0.0237 |
| hsa-miR-374b-3p | 2.98 | up | 11 | 32 | 0.0420 |
| hsa-miR-339-5p | 1.39 | up | 168 | 234 | 0.0476 |
| hsa-miR-10400-5p | 0.33 | down | 84 | 27 | 0.0496 |

**Exo_Con** = **Pd-Exos** (referring to exosomes obtained from placental explants cultured in complete culture medium);

**Exo_LPS** = **1-LPS-Pd-Exos** (referring to exosomes obtained from placental explants co-cultured with complete culture medium containing 1μg/mL LPS)

# Figures

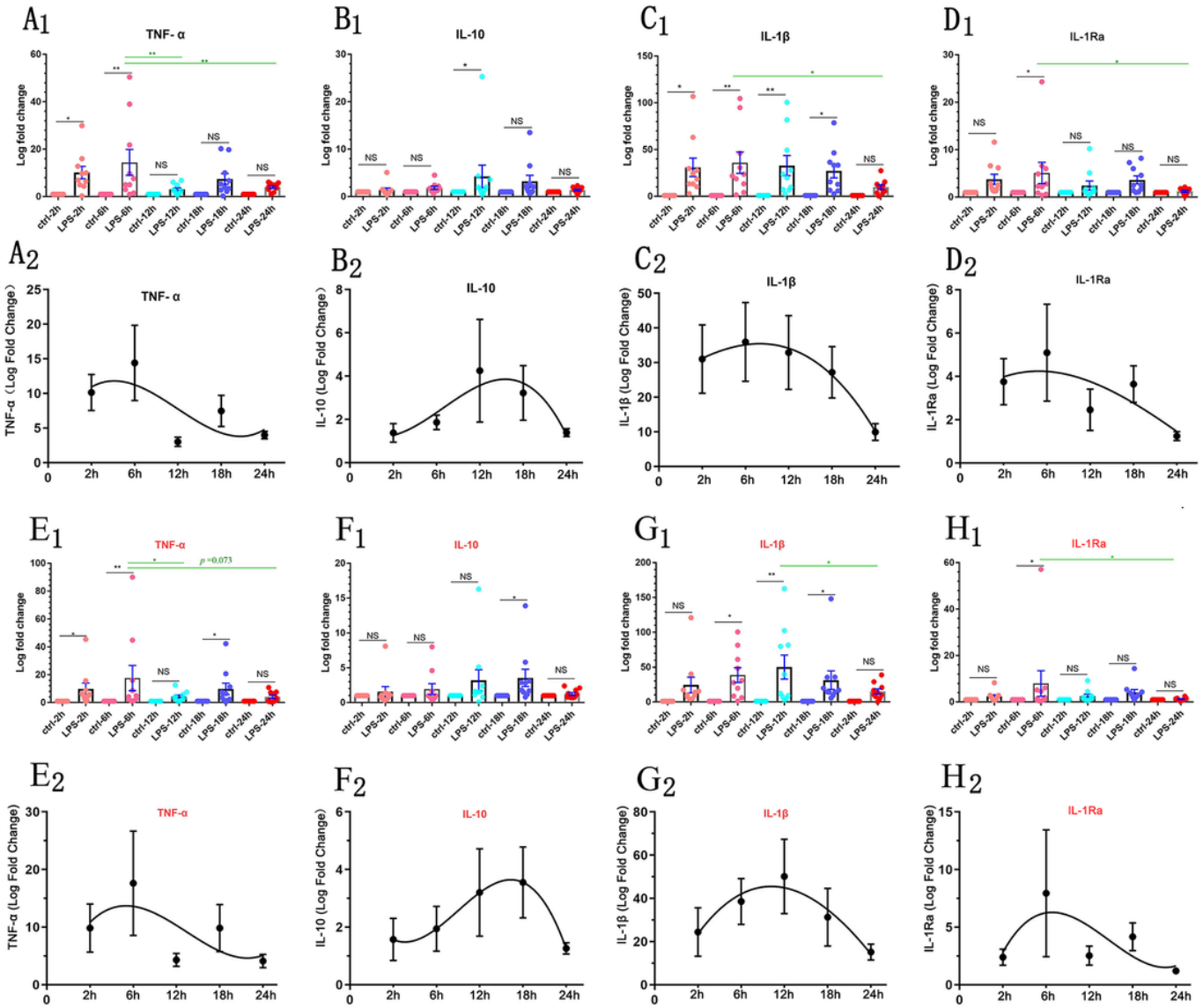



**Figure 1**

**RT-qPCR analysis of the effect of different concentrations of LPS on the expression of inflammatory genes in placental explants**

**($A_1$-$D_1$)** RT-qPCR analysis was conducted to investigate the effect of 1μg/ml LPS on the expression of inflammatory genes in placental explants (n=10). **($E_1$-$H_1$)** (placental explants treated with 10μg/mL LPS).

Note: Different time points of LPS treatment groups were each compared to the control group cultured in complete medium at the same time. (Black lines represent intragroup comparisons; green lines represent intergroup comparisons; Pro-inflammatory cytokines: TNF-α, IL-1β; Anti-inflammatory cytokines: IL-10, IL-1Ra; Ctrl: control group). **($A_2$-$D_2$)** and **($E_2$-$H_2$)** represents the trend lines generated for the respective indices in (**$A_1$-$D_1$**) and (**$E_1$-$H_1$**) . * $p < 0.05$; ** $p < 0.01$; NS: not statistically significantThe data were presented as mean ± standard error.

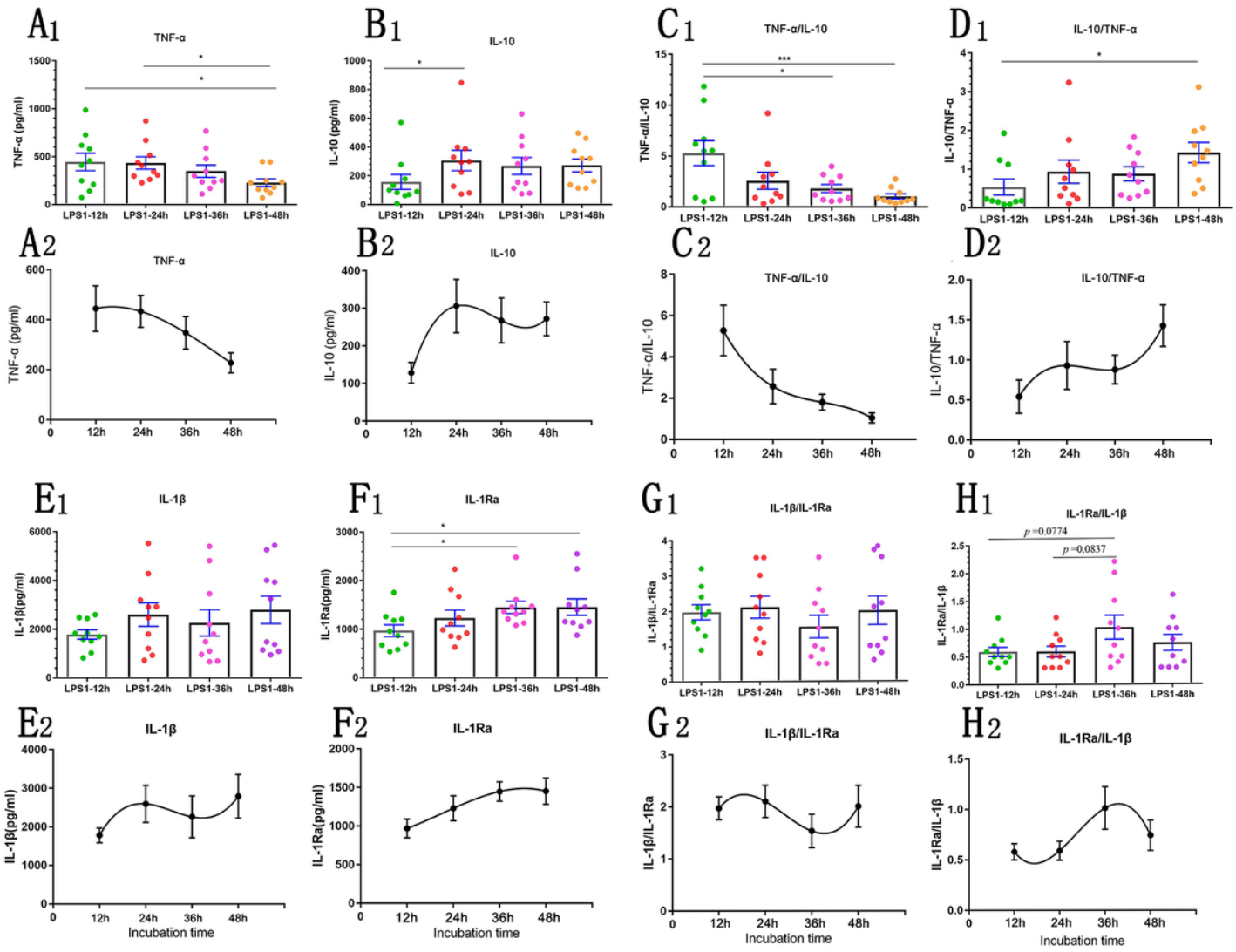
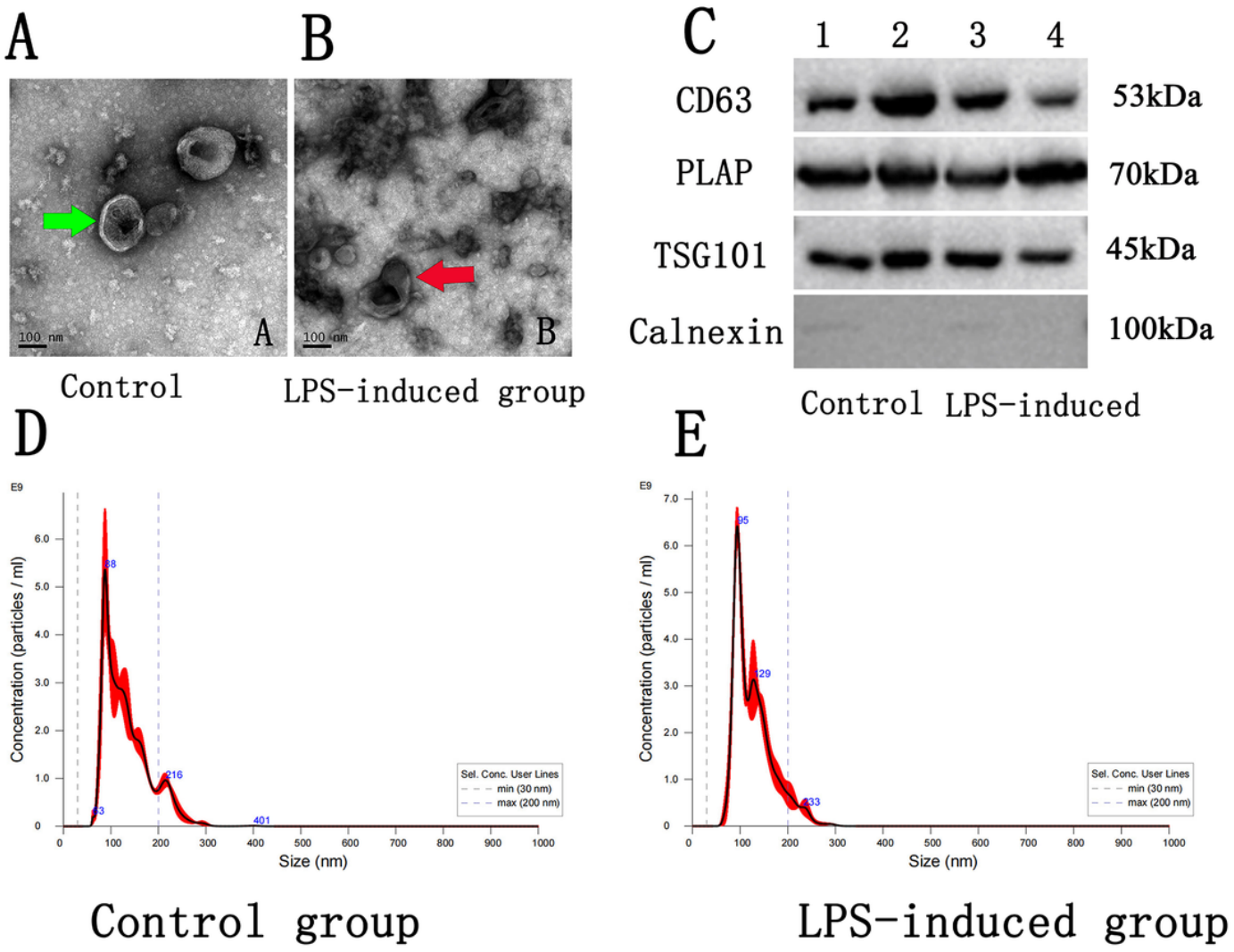



Figure 2

## ELISA analysis of the effect of LPS on the expression of inflammatory cytokines in the supernatant of placental explant cultures

**($A_1$-$D_1$)** After co-culturing 1μg/mL LPS with placental explants for 12h, 24h, 36h, and 48h, the expression trends of TNF-α and IL-10 inflammatory factors, as well as the ratio between the two factors, were analyzed in the culture supernatant. **($E_1$-$H_1$)** Additionally, the expression trends of IL-1β, IL-1Ra, and their ratio were also assessed. **($A_2$-$D_2$)** and **($E_2$-$H_2$)** represents the trend lines generated for the respective

indices in ($A_1$-$D_1$) and ($E_1$-$H_1$) . * $p < 0.05$; *** $p < 0.001$; The data were presented as mean ± standard error.




## Figure 3

**Identification of the two groups of placenta-derived exosomes (Pd-Exos) through transmission electron microscopy (TEM), western blot (WB), and nanoparticle tracking analysis (NTA) techniques**

**(A-B)** Transmission electron microscopy was used to characterize the extracted and purified Pd-Exos from the two groups (scale bar = 100 nm). The green and red arrows indicate the exosomes, revealing saucer-like structures. **(C)** Western Blot was performed to analyze the surface protein markers (CD63, TSG101) of the two groups of Pd-Exos. PLAP was identified as a characteristic protein of Pd-Exos, and Calnexin served as the negative control. **(D-E)** Nanoparticle tracking analysis (NTA) was employed to characterize the size distribution of the two groups of exosomes, which was consistent with the typical exosome dimensions.

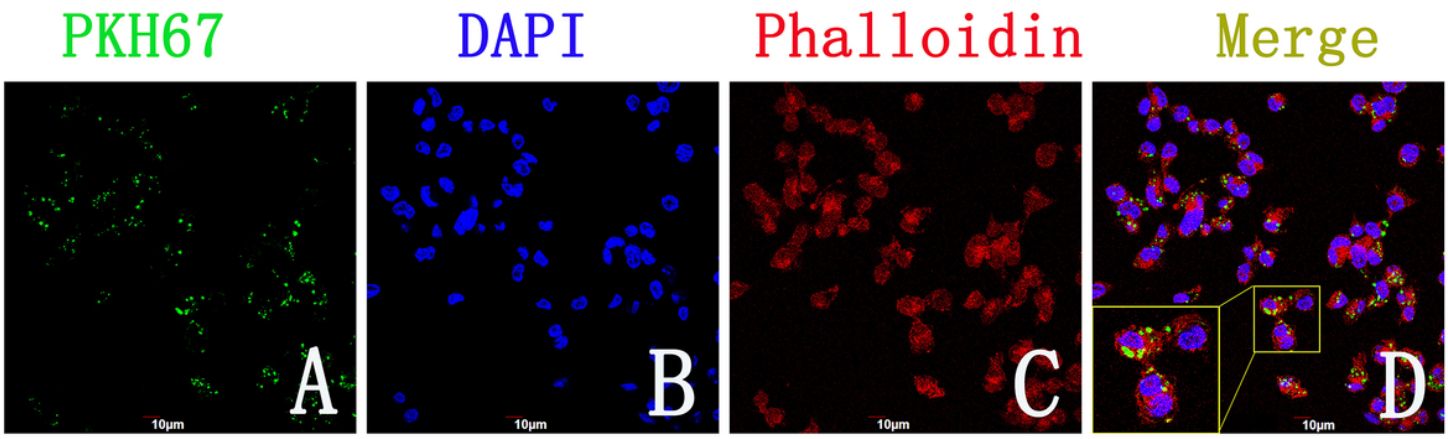



Figure 4

## PKH67-Labelled exosomes internalize by THP-1 cells

PKH67-labeled Pd-Exos were internalized by differentiated THP-1 cells. Exosomes were labeled with green fluorescence, the cytoskeleton with red fluorescence, and the cell nuclei with blue fluorescence. Scale bar = 10 μm.

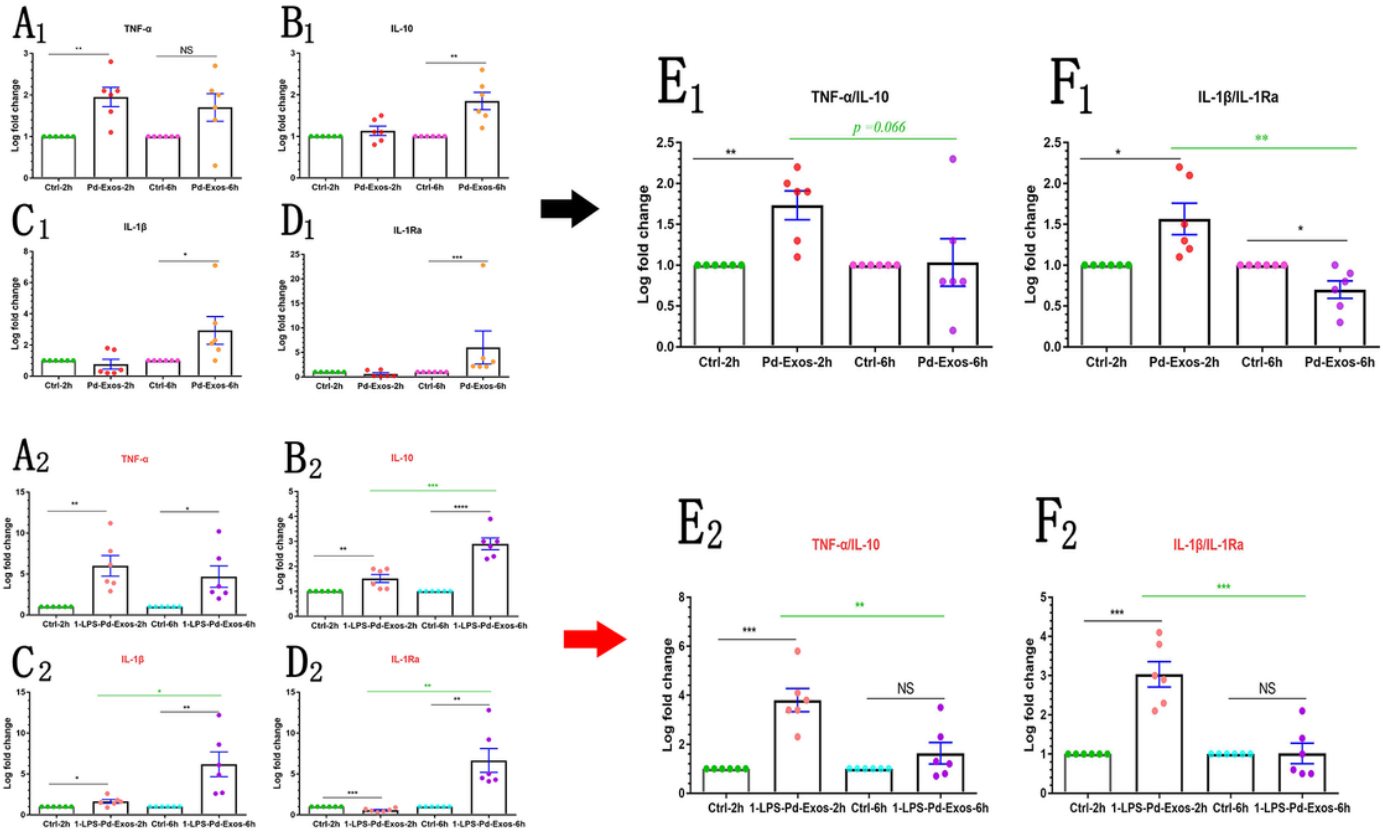



Figure 5

### Impact of Pd-Exos treatment on the expression of inflammatory genes in THP-1 cells

Analysis of the effects of 100ng/mL Pd-Exos or 100ng/mL 1-LPS-Pd-Exos the expression of inflammatory genes and the ratio of pro-inflammatory to anti-inflammatory factors in THP-1 cells using RT-qPCR. The treatment durations were 2 hours and 6 hours. The following parameters were assessed: TNF-α, IL-10, IL-1β, IL-1Ra, TNFα/IL-10 ratio, and IL-1β/IL-1Ra ratio. **($A_1$-$F_1$)** represents THP-1 cell line treated with 100ng/mL Pd-Exos, while **($A_2$-$F_2$)** represents THP-1 cell line treated with 100ng/mL 1-LPS-Pd-Exos. (Black lines represent intragroup comparisons; green lines represent intergroup comparisons, with data presented as mean ± standard error; Ctrl: control group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS: not statistically significant).

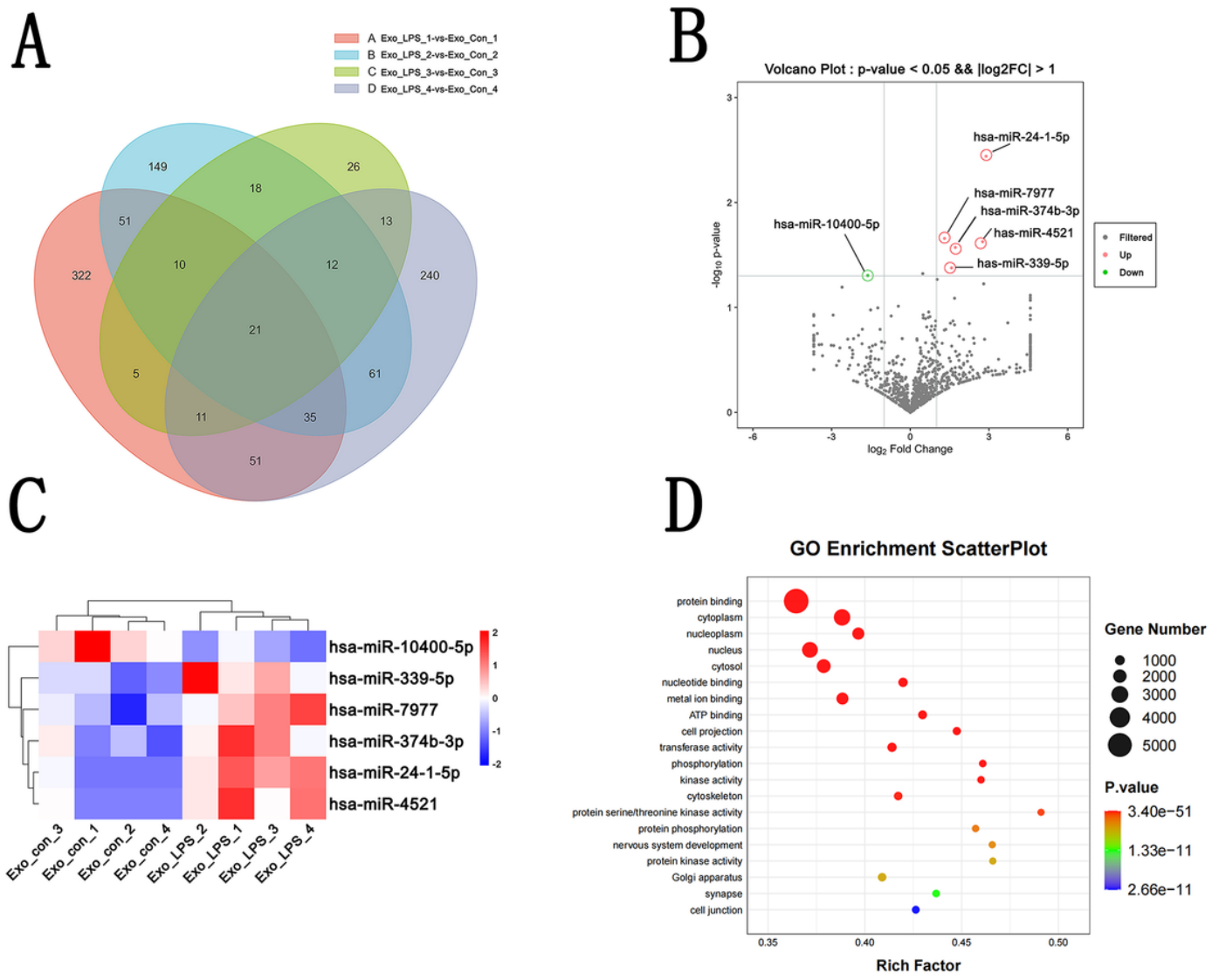
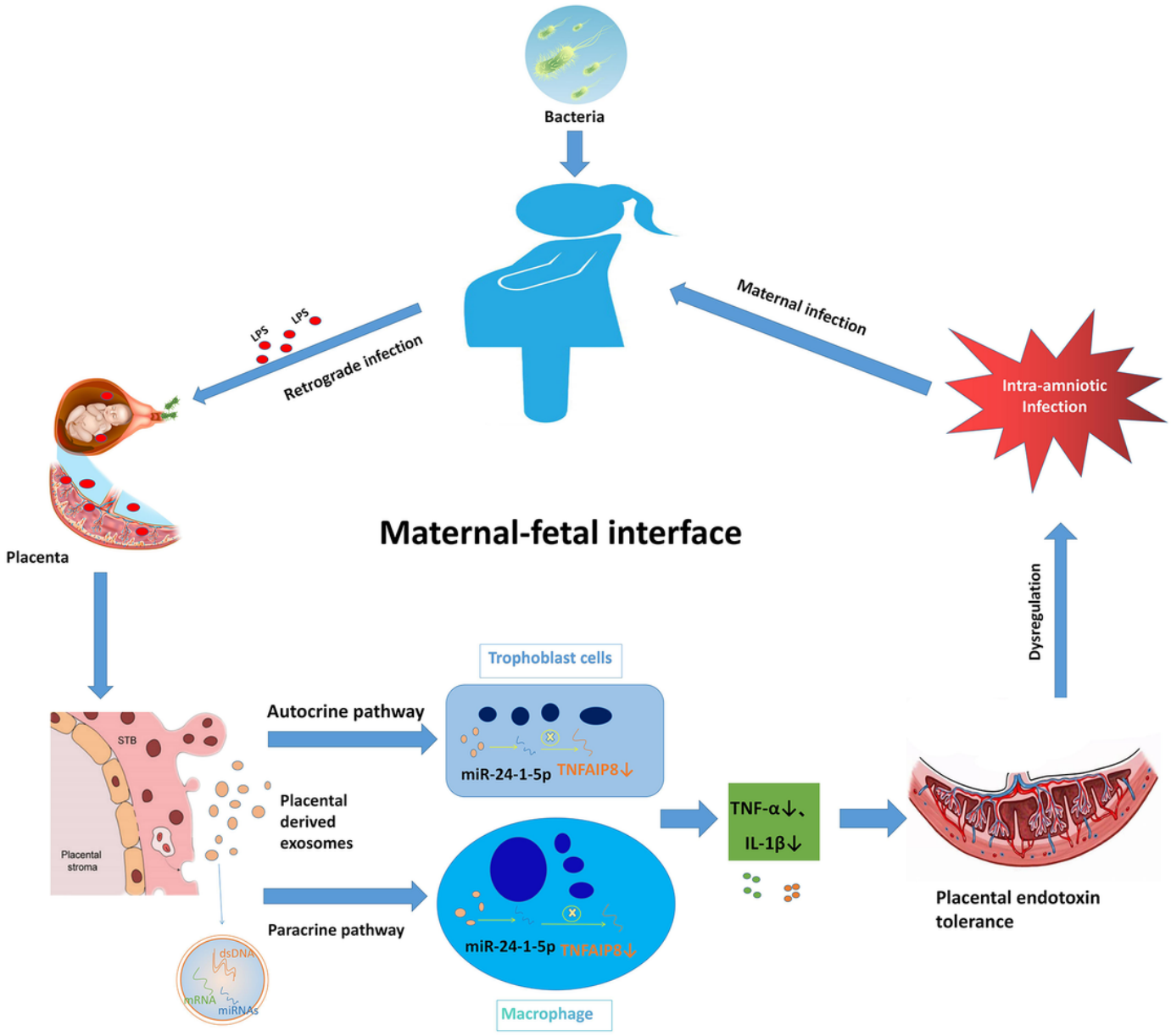



Figure 6

### Screening of differentially expressed placenta-derived exosomal miRNAs related to placental endotoxin tolerance

**(A)** Venn diagram depicting 1025 differentially expressed miRNAs among 4 pairs of placenta-derived exosome samples. This diagram illustrates the shared and overlapping differentially expressed miRNAs among the 4 exosome samples. **(B)** Volcano plot displaying the differentially expressed miRNAs in 4 pairs of placenta-derived exosomes, with miR-24-1-5p being the most significantly differentially expressed miRNA. **(C)** Hierarchical clustering analysis of the selected significantly differentially expressed miRNAs using a heat map. **(D)**GO enrichment analysis investigating the enrichment of target genes regulated by the differentially expressed miRNAs.




## Figure 7

**Schematic diagram of the mechanism of placenta-derived exosomal miR-24-1-5p involvement in the regulation of inflammation at the maternal-fetal interface**

In LPS-induced placental explants, the expression of miR-24-1-5p is upregulated in Pd-Exos produced. These exosomes and their carried miRNA, through paracrine pathway (interacting with maternal-fetal interface macrophages) and autocrine pathway (interacting with trophoblasts themselves), are internalized by recipient cells. Upon internalization, they inhibit the expression of TNFAIP8 in recipient cells, thereby suppressing the downstream inflammatory factors TNF-α and IL-1β, and participating in placental endotoxin tolerance. If the placenta's tolerance to endotoxin becomes impaired, it may lead to secondary IAI, resulting in subsequent maternal-fetal complications.

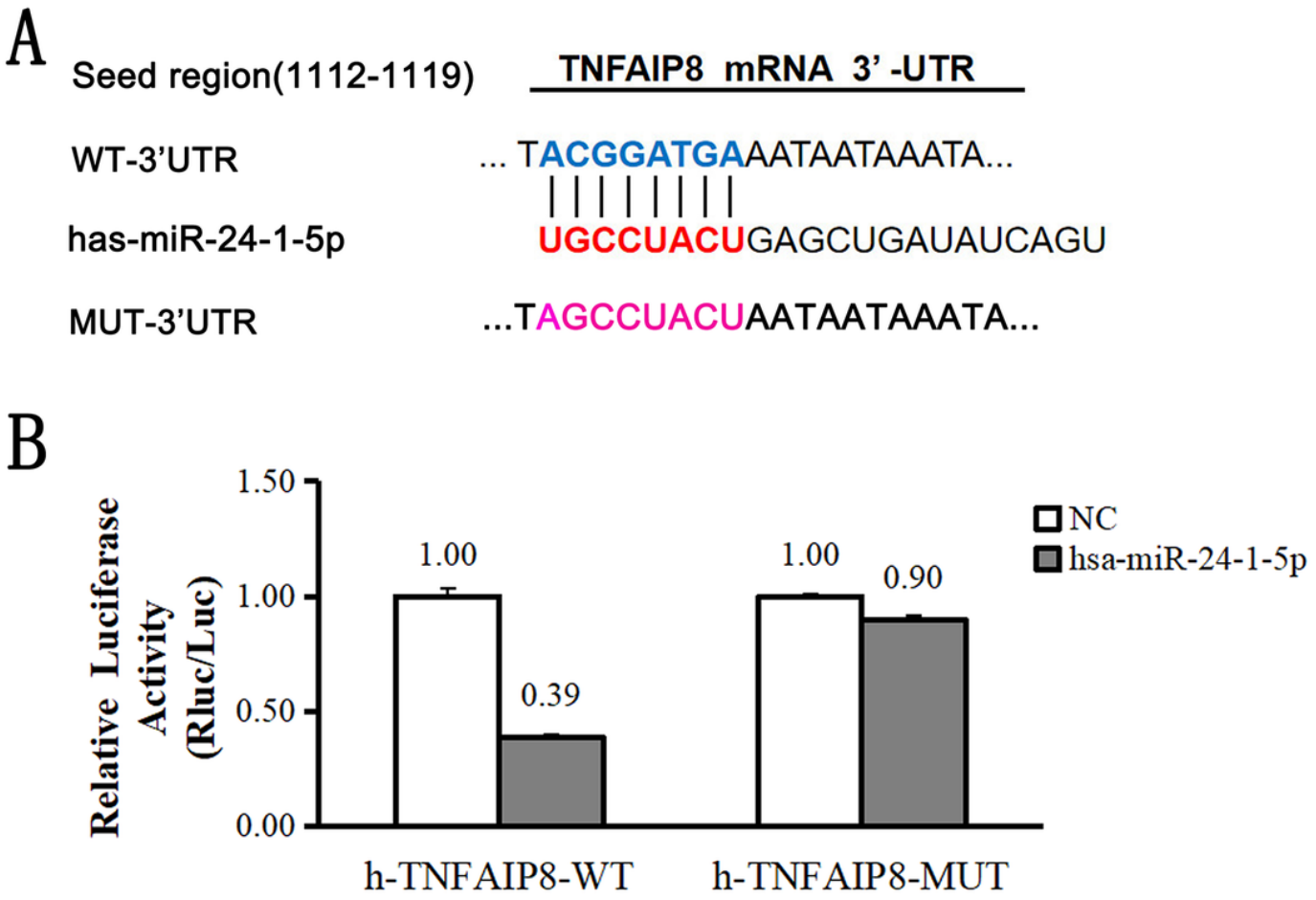
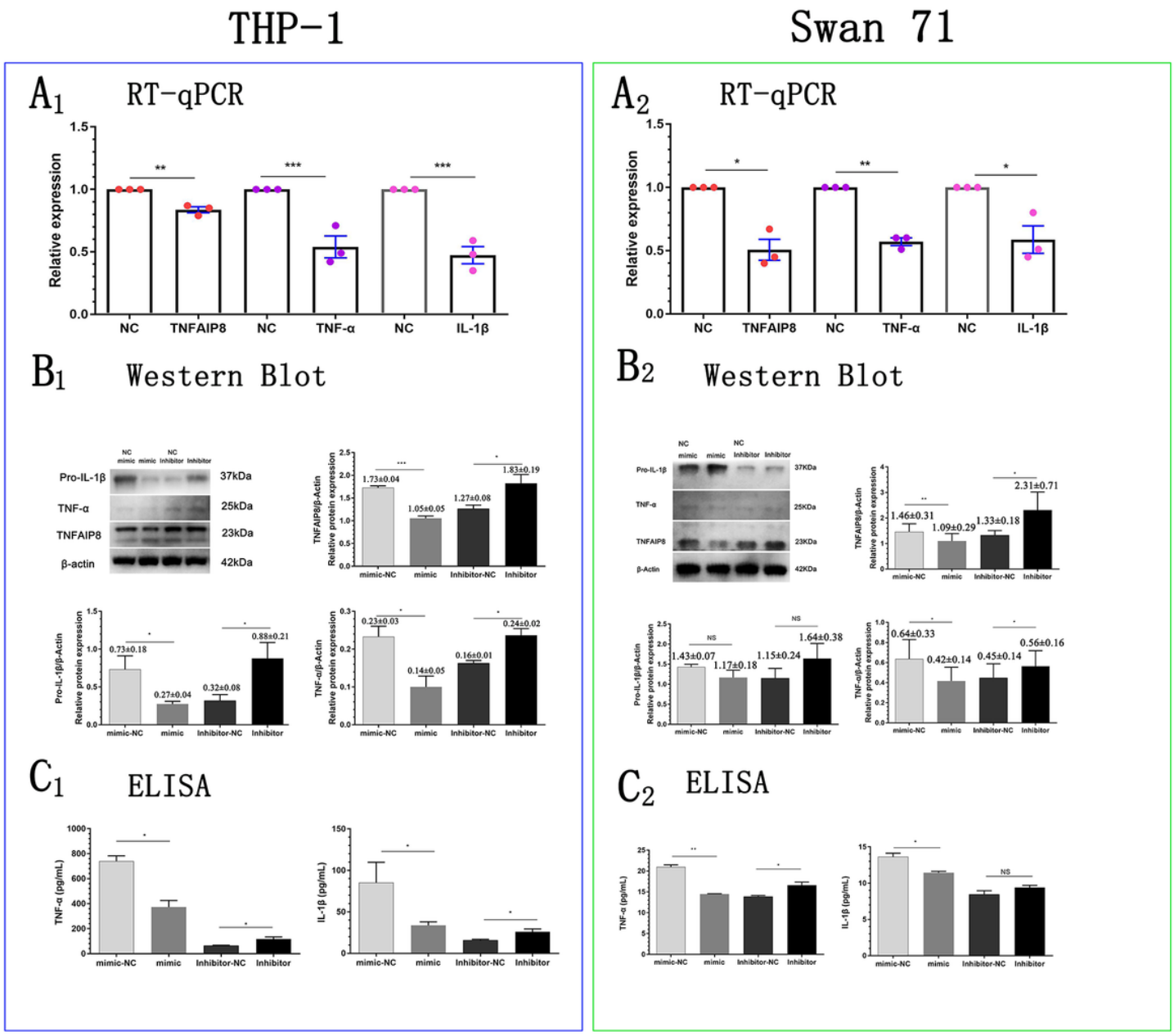



**Figure 8**

**The dual-luciferase reporter gene analysis confirmed the targeting relationship between miR-24-1-5p and the target gene TNFAIP8.**

(A) Schematic diagram of the putative miR-24-1-5p-binding site in the 3'UTR region of TNFAIP8 mRNA. The seed sequence of miR-24-1-5p matches the 3'UTR region of TNFAIP8 mRNA (in red). The mutated sites in the 3'UTR region of TNFAIP8 are highlighted in pink (7 nucleotides underwent mutation). **(B)** Dual luciferase assay on wild-type (WT) or mutant TNFAIP8 3'UTR in 293T cells transfected with mimic negative control (NC) or miR-24-1-5p, and calculated as the luciferase activity ratio of firefly to Renilla luciferase. The results are presented as the mean ± standard deviation. ***$p$ <0.001 (Student's t-test).




## Figure 9

### Effect of miR-24-1-5p Overexpression in Recipient Cells on Target Genes and Downstream Inflammatory Cytokine Expression

**($A_1$)** THP-1 cells were transfected with 100nmol/L miR-24-1-5p mimic, and the expression of the target gene TNFAIP8 and downstream inflammatory factors TNF-α and IL-1β was analyzed by RT-qPCR. **($B_1$)** THP-1 cells were transfected with 100nmol/L miR-24-1-5p mimic or inhibitor, along with their corresponding negative controls. The expression of TNFAIP8, TNF-α, and IL-1β precursors was monitored using Western blot. **($C_1$)** ELISA was performed to analyze the levels of inflammatory factors TNF-α and IL-1β in the cell culture supernatant of THP-1 cells transfected with 100nmol/L miR-24-1-5p mimic, inhibitor, and their corresponding negative controls. **($A_2$-$C_2$)** Swan 71 cells were transfected with 100nmol/L miR-

24-1-5p mimic, inhibitor, and their corresponding negative controls. The expression changes of TNFAIP8 and downstream inflammatory factors TNF-α and IL-1β were analyzed by RT-qPCR, Western blot, and ELISA, respectively. Data were obtained from three independent experiments and presented as the mean ± standard error. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS: not statistically significant.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.tif
- FigS2.tif
- Supplementarytables.docx