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The Regulatory Role of Decidual RHOQ Downregulation on Aberrant Dendritic Cell Activation in Miscarriage

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Article

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Abstract

Purpose

Recurrent miscarriage (RSA) has an unexplained etiology in 52.4% of cases. Accurate prediction contributes significantly to improving pregnancy success rates while reducing gestational complications. This study identifies RHOQ as a predictor of abnormal immune status in miscarriage.

Methods

Key hub genes related to defective endometrial receptivity (DER) were identified through bioinformatic analysis. CIBERSORTx was used to assess immune infiltration status in DER patients and controls. Additionally, enrichment analyses were conducted to explore the pathways involved in DER. The immunoreactive score (IRS) was used to verify RHOQ expression and dendritic cell numbers in clinical samples. ELISA was used to measure the expression of inflammatory factors across different groups. Western blotting was used to measure CD40 and CD83 protein levels.

Results

The proportion of mature dendritic cells was significantly increased in patients with DER. RHOQ levels in decidual tissue from the DER group were markedly lower than those in the normal control (NC) group and negatively correlated with the number of mature dendritic cells. Clinical samples confirmed that the number of mature dendritic cells was greater in the RSA group than in the NC group. RHOQ expression was lower in decidual tissue from the RSA group and negatively correlated with the number of mature dendritic cells. The downregulation of RHOQ in human endometrial cells leads to DC maturation. Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) were upregulated in an RHOQ-silenced human endometrial epithelial cell line.

Conclusion

RHOQ can serve as a reliable biomarker for detecting abnormal immune status during pregnancy.

Clinical trial registration

This experiment is not a clinical trial.

Introduction

Recurrent spontaneous abortion (RSA), defined as two or more consecutive miscarriages with the same sexual partner, has an incidence of 1% to 2% among all couples trying to conceive.(Deng et al. 2022). The primary causes of early recurrent miscarriages include chromosomal abnormalities of the embryo, immune dysfunction, luteal insufficiency, and hypothyroidism; however, the etiology remains unclear in 52.4% of cases, and 80% of unexplained abortions are closely associated with immune-related factors (La et al. 2021).

Immune imbalance is closely associated with unexplained RSA (Li D et al. 2021). Specifically, an imbalance in the immune microenvironment of the maternal–fetal interface can lead to a failure to maintain maternal immune tolerance toward the embryo, which represents one of the critical causes of RSA. Compared with age-matched healthy controls, women with RSA exhibit lower levels of the tissue-resident marker CD49a on decidual natural killer (dNK) cells and higher perforin, granzyme B, and IFN-γ levels (Zhang X,et al. 2021). Decidual natural killer (dNK) cells play crucial roles in regulating trophoblast invasion and spiral artery remodeling. Moreover, T cells and dendritic cells have been recognized as key mediators of immune homeostasis at the maternal–fetal interface. Although dendritic cells constitute only 1%-2% of the total immune cells at the maternal–fetal interface, they play a pivotal role in the local immune regulatory system of the uterus during pregnancy, particularly in mediating communication between innate and adaptive immunity (Mahajan et al. 2024). Additionally, dendritic cells (DCs) interact with immune cells such as T cells, NK cells, and macrophages, and respond to the dynamic changes in hormone levels that occur during pregnancy (N.M. Shah et al. 2017, E. Leno-Duran et al.2014, E. Tagliani et al. 2008, R.R. Nair et al. 2017). Abnormal DC differentiation or function has been linked to pregnancy complications, including recurrent spontaneous abortion (RSA), preterm birth (PTB), preeclampsia (PE), peripartum cardiomyopathy (PPCM), and infectious obstetric disorders (S. Liu et al.2018, H.Q. Lu et al. 2019, M. Le Gars et al. 2012). DCs are classified as immature (imDCs) or mature (mDCs). In pregnant women, imDCs dominate in the decidua due to rapid imDC expansion and stable mDC levels (D. Aldebert et al. 2007, C. Bartmann et al. 2014), reflecting functional adaptations that support immune tolerance. Decidual mDCs produce less IFN-γ, TNF-α, and IL-15 than imDCs do and exhibit weaker NK cell stimulation (V. Bachy et al. 2008). Decidual mDCs also produce less IL-12 and promote Th2 responses to a lesser extent than peripheral blood mDCs do (S. Miyazaki et al. 2003). Overall, the immature DC phenotype supports maternal–fetal tolerance, but the regulatory mechanisms remain unclear.

Collectively, the immature phenotype of dendritic cells (DCs) appears to support maternal–fetal tolerance. However, the underlying regulatory mechanisms remain incompletely understood, and studies investigating the factors driving this aberrant differentiation are limited. In RSA research, several studies have utilized GEO database mining to identify hub genes; however, due to small sample sizes, the findings have been inconsistent (Chen et al. 2019). To identify molecular markers that can predict abnormal immune states and adverse pregnancy outcomes, we systematically integrated four datasets (GSE58144, GSE165004, GSE92324, and GSE76862) to detect immune cell populations with differential abundance and genes with differential expression between DER and control group. Using screening and correlation analysis, we revealed that RHOQ was significantly downregulated in DER and correlated with mature dendritic cell expression. RHOQ is a member of the Ras superfamily of small GTPases. Previous

studies have demonstrated that RHOQ activates STAT3 signaling, which plays a pivotal role in the abnormal differentiation of dendritic cells (DCs) associated with recurrent spontaneous abortion (RSA) (Debidda et al. 2005). Clinical samples were used to validate the differences in RHOQ expression and the number of dendritic cells between the two groups. The primary objective of this study was to investigate whether RHOQ can serve as a reliable biomarker for detecting abnormal immune status during pregnancy, thereby enabling SA patients with such immune abnormalities to receive timely immunomodulatory interventions and ultimately improve pregnancy outcomes.

Methods

Subjects

RSA patients (RSA group, n = 40) and normal pregnant women (control group, n = 20) recruited from the Fifth Affiliated Hospital of Guangzhou Medical University from January to December 2023 were included in this study. Written informed consent was obtained from all participants prior to undergoing artificial abortion. Data, including baseline demographic and clinical characteristics of the enrolled patients, specifically age, gravidity, parity, history of miscarriage, body mass index (BMI), and gestational age at the time of pregnancy termination, as well as other relevant clinical indicators, were collected. The inclusion criteria included patients diagnosed on the basis of at least two consecutive spontaneous abortions confirmed by imaging findings and pathological evidence during the first trimester; singleton intrauterine pregnancy; and good patient compliance. Exclusion criteria included the following: (1) a history of endocrine disorders; (2) the presence of reproductive tract infection; (3) the use of immune-modulating agents or endocrine-disrupting drugs within the last three months; (4) an abnormal chromosome karyotype in either the patient or her partner; (5) concomitant medical conditions during pregnancy, such as tumors or hepatitis; and (6) incomplete clinical documentation. The subjects in the control group had no history of spontaneous abortion, stillbirth, or preterm labor. This study was approved by the Ethics Committee of the Fifth Affiliated Hospital of Guangzhou Medical University (KY01-2021-09-08).

Procedure

Microarray data

Microarray datasets were systematically retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) using the clinically and terminologically aligned search terms “recurrent spontaneous abortion”, “recurrent pregnancy loss”, and “habitual abortion”. This strategy yielded four eligible, publicly available datasets: GSE58144, GSE165004, GSE92324, and GSE76862. GSE58144 comprises luteal-phase endometrial biopsies from 43 patients with recurrent implantation failure (RIF) and 72 fertile controls (FC). GSE165004 includes endometrial samples from 24 patients with recurrent miscarriage (RM), 24 with unexplained infertility (UI), and 24 fertile controls. GSE92324

contains specimens from 10 RIF patients and 8 healthy donors with documented, uncomplicated singleton pregnancies (serving as non-RIF, pregnancy-confirmed controls). GSE76862 consists of endometrial tissues from three healthy controls (HC) and three RM patients. Patients with UI and RIF were intentionally included because defective endometrial receptivity (DER) is a mechanistically validated, shared etiological factor in both conditions—and a recognized contributor to early pregnancy loss. As DER disrupts critical embryo–endometrium crosstalk during the window of implantation, integrative immune and transcriptomic comparisons between DER-affected individuals and rigorously matched controls provide a biologically grounded framework for discovering predictive biomarkers of spontaneous abortion. Collectively, these four datasets encompass 104 DER-affected individuals (comprising RIF, RM, and UI patients) and 107 phenotypically well-characterized control subjects.

Identification of DEGs

The matrix files from the four datasets were processed, integrated, and analyzed using R software (version 3.6.2). Data from the four databases were merged, and differentially expressed genes (DEGs) were identified by utilizing the “DESeq2” package in R (version 4.1.3), with thresholds set at |log2 FC| > 1 and adjusted P value < 0.05. The “ggpubr” and “pheatmap” packages within R were employed to generate volcano plots and heatmaps, respectively, of the DEGs.

Functional enrichment analysis

Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed using the “org.Hs.eg.db” package and “clusterProfiler” package in R software. The GO database is composed of biological processes (BPs), cellular components (CCs), and molecular functions (MFs). A P value threshold of < 0.05 was considered to indicate statistical significance.

Analysis of immune cell infiltration patterns

The relative proportions of 22 immune cell subtypes within the bulk tissue samples were estimated using CIBERSORTx, a deconvolution algorithm based on a validated leukocyte gene signature matrix (LM22). To mitigate technical heterogeneity arising from the integration of multiple microarray datasets (GSE58144, GSE165004, GSE92324, GSE76862), we applied the ComBat algorithm via the sva R package for empirical batch correction. The effectiveness of this correction in removing major platform-specific batch effects was confirmed by visual inspection of principal component analysis (PCA) plots before and after adjustment.Pairwise correlations between the expression levels of differentially expressed genes (DEGs) and the estimated abundances of immune cell types were calculated using the corrplot package in R, with statistical significance set at P < 0.05. To specifically evaluate the robustness of the key negative correlation observed between RHOQ expression and mature dendritic cell abundance, we conducted a leave-one-dataset-out sensitivity analysis. This involved iteratively excluding each of the

four source datasets and recalculating the correlation coefficient. The persistence of a significant negative correlation ($P < 0.05$) in all four subset analyses confirmed that this association was not an artifact driven by any single cohort, thereby strengthening the reliability of the finding.

Immunohistochemical staining

Immunohistochemical staining was employed to assess the differences in dendritic cell numbers and RHOQ expression levels between the two groups. Decidual tissues from the two groups were fixed in 10% formalin, embedded in paraffin, and subjected to immunohistochemical staining. Following blocking with bovine serum albumin (BSA), the sections were incubated with primary antibodies against RHOQ (1:200, PA5-102866, rabbit, Invitrogen, USA), CD 83 (1:500, ab275021, rabbit IgG, Abcam, USA) and CD 1a (ab268142, rabbit IgG, Abcam, USA) overnight at 4°C, followed by incubation with a goat anti-rabbit IgG secondary antibody (cat. no. #S0001; 1:200; Affinity) at 37°C for 1 hour. Immunohistochemical images were then captured using an optical microscope. The immunoreactive score (IRS) was calculated on the basis of the following criteria. (1) The number of positive cells in five randomly selected high-power fields was counted. Scores were assigned as follows: 1 point for 0%–10% positivity, 2 points for 10%–50% positivity, 3 points for 50%–75% positivity, and 4 points for 75%–100% positivity. (2) Staining intensity was scored as follows: 0 for negative samples, 1 for weak (light yellow) staining, 2 for moderate staining, and 3 for strong staining.

Cell culture

Due to the central role of endometrial epithelial cells as direct functional effectors of endometrial receptivity, we selected a well-characterized human endometrial epithelial cell line (EECs; BC-PC-HU-004, Nanjing Shenghang Biotechnology Co., Ltd., China) for subsequent experiments to minimize confounding effects arising from tissue heterogeneity and thereby enhance biomarker specificity, we also included a decidualized cell line (GNM51, National Collection of Authenticated Cell Cultures, China). Both cell lines were cultured in RPMI-1640 medium (Gibco, Billings, MT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat. No. 16000044) under a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection

To knock down RHOQ, short hairpin RNA (shRNA) constructs were chemically synthesized by Guangzhou Boyao Biological Co., Ltd. (Guangzhou, China): RHOQ-sh (F: GAAACUAGCAAAAGAGAUATT, R: UAUCUCUUUUGCUAGUUUCTT) and a scrambled negative control shRNA (NC shRNA; F: UUCUCCGAACGUGUCACGUTT, R: ACGUGACACGUUCGGAGAATT). The shRNA sequences were then cloned and inserted into recombinant shRNA expression vectors using Lipofectamine 2000 (Invitrogen). After transfection, the supernatants were collected 72 hours later. To generate stably transfected cell lines, infected human endometrial epithelial cells were selected by incubation with 4 μg/mL puromycin (Invitrogen) for two weeks.DCs are typically stimulated with Escherichia coli lipopolysaccharide (LPS) at

a concentration of 1 μg/mL for 18 hours to serve as a positive control (LPS group)—consistent with established experimental protocols reported in the literature(Baert T, et al, 2016).

Cell Culture and Coculture

Human endometrial epithelial cells (hEECs, BC-PC-HU-004, Nanjing Shenghang Biotechnology Co., Ltd., China) and a human dendritic cell line (GNM51, National Collection of Authenticated Cell Cultures, China) were cultured in RPMI-1640 medium (Gibco, Billings, MT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, #16000044) at 37°C in a humidified atmosphere containing 5% CO_2.

For the coculture experiments, hEECs were first transfected with either RHOQ-targeting shRNA (si-RHOQ group) or a scrambled negative control shRNA (si-NC group) as described in the “Cell Transfection” section. 72 hours post-transfection, the culture medium was replaced with fresh RPMI-1640 containing 10% FBS. DCs were then added to the hEEC monolayers at a DC-to-hEEC ratio of 1:5. The cells were cocultured in direct contact for 24 hours to allow for cell-cell interaction. As a positive control for DC maturation, a separate group of DCs was stimulated with LPS(1 μg/mL) for 18 hours (LPS group). Following the coculture period, the supernatants were collected, centrifuged to remove cellular debris, and stored at -80°C for subsequent cytokine analysis by ELISA. The cocultured DCs were carefully harvested for protein extraction and Western blot analysis to assess maturation markers.

Enzyme-linked immunosorbent assay (ELISA)

Tumor necrosis factor alpha (TNF-a) and interleukin 6 (IL-6) ELISA kits(CHUNDU. WuHan, China) were used according to the manufacturers’ instructions to measure the levels of inflammation-associated cytokines in the uman endometrial epithelial cell lines. The cell culture medium for ELISA was prepared by collecting supernatants from co-cultured human endometrial epithelial cell lines. The medium was centrifuged at 1200 rpm for 10 minutes to remove cellular debris, and the supernatant was carefully aspirated and stored at -80°C until analysis. For normalization, the total protein concentration of each medium sample was determined using a BCA kit (Vazyme, Nanjing, China) following the manufacturer's protocol. Briefly, 25 μL of each medium sample or protein standard was mixed with 200 μL of BCA working reagent, incubated at 37°C for 30 minutes, and the absorbance was measured at 450 nm. The total protein concentration was calculated from the standard curve, A linear regression analysis was performed using the standard samples, with concentration (x-axis) plotted against the corresponding optical density (OD) values (y-axis) in an Excel worksheet. Sample concentrations were subsequently determined by applying the derived regression equation.Experiments were conducted with 3 independent biological replicates per group.

Western blotting

Proteins were extracted from the harvested DCs after coculturewith different conditions. The whole cellular extracts were obtained by lysing the cells using a RIPA buffer supplemented with protease and phosphatase inhibitors on ice, following standard protocols for adherent cell collection and lysis. The lysates were then separated by SDS—PAGE, and the proteins were subsequently transferred onto PVDF

membranes. The membranes were incubated with primary antibodies according to the manufacturer's instructions (CD40, 1:1000; ab252557; Abcam, USA; CD83, 1:1000; ab244204; Abcam, USA) for 3 h, followed by incubation with a secondary antibody (BA1051; Boster, USA) for 1 h. β-Actin (BM0627; Boster, USA) served as the loading control. Chemiluminescence detection was performed using an enhanced chemiluminescence (ECL) protein blotting detection kit (human IgG) (P1015; Solarbio, China).Experiments were conducted with 3 independent biological replicates per group.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. Continuous variables following a normal distribution are presented as the mean ± standard deviation, and intergroup differences were assessed using one-way ANOVA. Paired t tests were conducted for normally distributed paired data, whereas chi-square tests were applied for categorical variables. Pearson correlation analysis was performed to evaluate the correlations between gene expression and immune cell abundance, The clinical parametersages (BMI, gestational age at pregnancy termination, gravidity/parity) and the IRS value of RHOQ expression were included as independent variables in a multiple linear regression model to control for confounding factors. $P < 0.05$ was considered to indicate statistical significance.

Results

The DEGs were identified from the GEO datasets.

To identify the DEGs between RSA patients and normal controls, we integrated data from four GEO databases (GSE58144, GSE165004, GSE92324, and GSE76862) and performed statistical analysis using R software. The data from the four datasets were integrated using R software (detailed database information is presented in Table 1), and batch effects across the datasets were systematically removed. This analysis included a total of 104 RSA patients and 107 patients with normal pregnancies. With R packages "limma" and "sva", four datasets were then merged and batch-normalized. Principal component analysis (PCA) was conducted on the processed data to assess the overall variation (Fig. 1a). Statistically significant differentially expressed genes ($P < 0.05$) were identified, resulting in a total of 2506 genes, comprising 1153 upregulated and 1353 downregulated genes. A volcano plot (Fig. 1b) was constructed to visualize all the DEGs, including both overexpressed and suppressed genes, whereas a heatmap (Fig. 1c) was generated to highlight the top 100 genes according to their expression levels (see Supplementary Table 1 for details).

Table 1
Data from the GSE58144, GSE165004, GSE92324, and GSE76862 datasets

GEO dataset	Region	Platform	Sample size
GSE58144	Endometrium	GPL15789	43 vs. 72 (CON)
GSE165004	Endometrium	GPL16699	48 vs. 24 (CON)
GSE92324	Endometrium	GPL10558	10 vs. 8 (CON)
GSE76862	Endometrium	GPL17586	3 vs. 3 (CON)

Figure 1 Genes that were differentially expressed between the RSA group and the normal pregnancy group were screened. 1a PCA plot after integration of the four databases. 1b Volcano plot of genes that are differentially expressed between RSA patients and patients with normal pregnancies. 1c Heatmap of the top 100 genes that are differentially expressed between RSA patients and patients with normal pregnancies. *P < 0.05

The biological functions and pathways related to the DEGs were revealed via functional enrichment analysis

We conducted functional enrichment analysis of the DEGs identified in the previous step to determine the possible biological functions and signaling pathways they may be involved in. The enrichment terms of Disease Ontology enrichment analysis(DO) revealed that the DEGs were enriched primarily in molecular biological processes, the top 30 biological processes included the regulation of autophagy, positive regulation of cellular catabolic processes, establishment of protein organelle localization, small GTPase-mediated signal transduction, and regulation of protein localization at the plasma membrane(Fig. 2a). The enrichment terms of KEGG demonstrated that these DEGs were associated with physiological processes such as oxidative phosphorylation, the cGMP-PKG signaling pathway, protein processing in the endoplasmic reticulum, and endocytosis(Fig. 2c). Furthermore, The enrichment terms of DO analysis revealed that the DEGs were significantly linked to gynecological diseases, such as placenta accreta and uterine sarcoma (Fig. 2b and Supplementary Tables 2–4).

The proportion of mature dendritic cells in the RSA group was higher than that in the normal group and was negatively correlated with the expression of RHOQ

We utilized CIBERSORTx (http://cibersortx.stanford.edu) to compare the abundance of 22 types of immune cells between the RSA group and the normal pregnancy group. The results demonstrated that the proportion of mature dendritic cells was greater in the RSA group than in the normal pregnancy group (Fig. 3a and b). Further investigation into the correlation between DEGs and mature dendritic cells revealed that the expression of the RHOQ gene was negatively correlated with the number of mature dendritic cells. Additionally, RHOQ expression was downregulated in the RSA group (Fig. 3c and d).

GO enrichment analysis revealed that RHOQ is significantly involved in key biological processes, including small GTPase-mediated signal transduction. This bioinformatic prediction aligns with established roles of Rho GTPases in immune regulation. For instance, a prior study demonstrated that RHOQ can activate signal transducer and activator of transcription 3 (STAT3) signaling, a pathway pivotal for the differentiation and function of DCs(Debidda et al. 2005). Furthermore, it is well-documented that members of the Rho family, orchestrate immune responses by activating the Rho-associated coiled-coil containing protein kinase (ROCK) signaling pathway, inhibition of the RhoA–ROCK axis has been shown to alleviate endometrial fibrosis and improve pregnancy outcomes in a mouse model (Xu et al. 2017). Integrating these mechanistic insights with our current GO analysis findings, we propose a novel hypothesis: The abnormal downregulation of RHOQ may disrupt the precise maturation program of dendritic cells within the uterine microenvironment, ultimately leading to local immune dysregulation.

The expression of mature dendritic cells (CD83+) was higher in the decidual tissue of the RSA group than in that of the normal pregnancy group

We assessed the IRS scores of CD83 and CD1a in patients with recurrent miscarriage and those with normal pregnancies to evaluate the differences in the expression levels of mature and immature dendritic cells between the two groups. A total of 60 patients who underwent induced abortion at the Fifth Affiliated Hospital of Guangzhou Medical University were enrolled and divided into two groups: the RSA group (n = 40) and the normal pregnancy group (n = 20). Decidual samples from these patients were subsequently collected for experiments. There were no significant differences in age, BMI, Gravidity times, Parity times and Gestational time since menopause between the two groups at baseline(Supplementary Table 5).The gestational age at pregnancy termination was 7.38 ± 1.07 weeks in the abortion group and 7.15 ± 1.34 weeks in the control group. Immunohistochemical analysis was performed on the decidual tissue from both groups to quantify the IRS of CD38 + cells(mature DCs) and CD 1a+ cells(immature DCs). IRS scores were calculated by multiplying the score for the percentage of positively stained cells by the score for the staining intensity. The IRS for mature dendritic cells (CD83+) was higher in the RSA group than in the normal pregnancy group ($P<0.05$) (Fig. 4).

RHOQ expression is decreased in RSA compared to normal pregnancies and exhibits a negative correlation with the abundance of mature dendritic cells

By analyzing the differences in the IRS results between the two groups, we observed that compared with that in the normal group, RHOQ expression in the decidua in the RSA group was markedly reduced. Additionally, the IRS value of mature dendritic cells was set as the dependent variable, while age, BMI, gestational age at pregnancy termination, gravidity/parity, and the IRS value of RHOQ expression were included as independent variables in a multiple linear regression model to control for confounding factors. After adjusting for these confounders, the results indicated that RHOQ expression was independently and negatively correlated with mature DC markers. The adjusted correlation coefficient was − 0.595 (95% CI: -0.730 to -0.413, $P < 0.001$, see Fig. 5).

Figure 5 Analysis of RHOQ expression intensity in the RSA group and normal pregnancy group. 5a, 5b and 5c Immunohistochemical staining and expression intensity analysis of RHOQ in the RSA group and normal pregnancy group. 5d Correlation analysis of RHOQ expression intensity in mature DC cells in the RSA group and normal pregnancy group. *P < 0.05, scale bar = 200 μm.

RHOQ downregulation in human endometrial epithelial cells leads to DC maturation

Human endometrial epithelial cells were transfected with either a blank plasmid and subsequently cocultured with DCs for 24 hours, which served as the control group (si-NC), or transfected with the si-RHOQ plasmid and cocultured with DCs (si-RHOQ group). Given that LPS (1 μg/ml) is known to promote DC maturation, the cells were cocultured with DCs as a positive control (LPS group). ELISAs revealed that the levels of the inflammatory cytokines IL-6 and TNF-α were significantly greater in the supernatants of si-RHOQ group than that in the si-NC group, and the expression pattern was similar to that observed in the LPS group (Fig. 6a and 6b). In contrast, the WB results demonstrated markedly greater CD40 and CD83 protein levels in the si-RHOQ group than in the si-NC group (Fig. 6c and 6d). Previous studies have demonstrated that IL-6 and TNF-α are secreted by dendritic cells following their activation, whereas CD40 and CD80 play critical roles in inducing dendritic cell maturation (Wei et al. 2022). On the basis of these findings, we hypothesize that downregulation of RHOQ in endometrial cells from patients with recurrent spontaneous abortion may induce abnormal activation of dendritic cells, increasing the secretion of IL-6 and TNF-α within the endometrial microenvironment, potentially contributing to the pathogenesis of recurrent spontaneous abortion.

Discussion

RSA poses significant risks to women’s reproductive health, including intrauterine adhesions, pelvic infection, infertility, and an increased risk of ectopic pregnancy, profoundly affecting the physical and psychological well-being of women of childbearing age (Dimitriadis et al., 2020). Therefore, elucidating the underlying mechanisms of RSA is of paramount importance. Advances in genomic technologies have highlighted the potential role of genetic factors in unexplained RSA, particularly genes involved in thrombotic and immunoregulatory pathways. Aberrant gene expression is closely associated with adverse pregnancy outcomes. For instance, dysregulation of WT1-associated proteins destabilizes early growth response protein 1, leading to ephrin receptor B4-mediated trophoblast dysfunction and contributing to RSA (Hua et al., 2024). Furthermore, genome-wide sequencing enables the identification of DEGs between RSA patients and women with normal pregnancies. Bioinformatics analysis of the GSE113790 dataset revealed that ZBTB24 is significantly downregulated in RSA decidua and impairs trophoblast proliferation, differentiation, and migration (Ruan et al., 2022). Such gene expression alterations can modulate immune cell activity, thereby disrupting the maintenance of pregnancy. For example, circular RNA CircDDX21 alleviates trophoblast dysfunction and promotes regulatory T-cell differentiation in RSA by modulating the miR-520a-5p/FOXP3/PD-L1 axis (Duan et al., 2024).

In the present study, integrated bioinformatics analysis of four RSA-related datasets from the GEO database revealed that RHOQ is downregulated in RSA decidua and shows a significant negative correlation with the estimated abundance of mature DCs. This observation is consistent with our subsequent validation using clinical samples, which confirmed the downregulation of RHOQ and the concomitant increase in mature DCs in RSA decidual tissues.GO enrichment analysis indicated that RHOQ is implicated in small GTPase-mediated signal transduction and the regulation of protein localization at the plasma membrane. As a member of the Cdc42 subfamily within the Rho family of small GTPases (Jahid et al., 2022), RHOQ belongs to a protein family known to regulate immune cell function. Previous studies have shown that Rho family GTPases, such as RhoA, modulate immune responses via the ROCK signaling pathway, and selective inhibition of the RhoA–ROCK axis alleviates endometrial fibrosis and improves pregnancy outcomes in mouse models of intrauterine adhesions (Xu et al., 2017). Additionally, Zhang et al. (2023) reported that decreased PD-1 levels in RSA patients suppress the RhoA-ROCK2 pathway, reducing decidual NK cell activity and contributing to pregnancy loss. Based on these established roles of Rho GTPases in endometrial immunology and our observed correlation between RHOQ and mature DCs, we hypothesize that the downregulation of RHOQ may be associated with disrupted DC maturation in the uterine microenvironment, potentially through mechanisms involving GTPase-dependent signaling. However, direct causal evidence linking RHOQ to specific downstream effectors (e.g., STAT3) in this context remains to be established and represents a key objective for future investigation.

The local immune microenvironment of the maternal uterus is critical for successful embryo implantation and pregnancy maintenance (Robertson et al., 2022). Disruption of this delicate balance can compromise maternal immune tolerance, leading to embryo rejection and pregnancy loss. Dendritic cells are key antigen-presenting cells that play a pivotal role in regulating immune responses and establishing maternal–fetal tolerance. An imbalance between mature and immature DC subsets can disrupt the Th1/Th2 equilibrium, contributing to adverse pregnancy outcomes (Negishi, Y., et al. 2012). In this study, by integrating four GEO datasets comprising 211 samples from DER patients and normal controls, we compared the immune cell composition in decidual tissues. The results demonstrated a significantly elevated proportion of mature DCs in DER decidua, suggesting a potential link between DC imbalance and RSA pathogenesis. This computational finding was further validated using clinical decidual samples, which confirmed the increased abundance of mature DCs in RSA patients compared to controls.However, since some of the data in the database are derived from RIF and normal healthy women, decidualized mid-luteal endometrial cells have a distinct phenotype from endometrial decidual cells of first-trimester pregnant women, the possibility of bias exists.

To explore the functional interaction between RHOQ and DCs, we performed an in vitro coculture assay. When DCs were cocultured with RHOQ-silenced human endometrial epithelial cells, the levels of the pro-inflammatory cytokines IL-6 and TNF-α in the supernatant were significantly increased compared to the si-NC control group, showing a pattern similar to that induced by LPS stimulation. Given that IL-6 and TNF-α are established markers of DC activation, these results suggest that RHOQ downregulation in endometrial cells may create a microenvironment conducive to DC muturation. Based on these collective

findings, we propose that RHOQ downregulation in the endometrium of RSA patients may be associated with aberrant DC muturation and elevated pro-inflammatory cytokine secretion, which could contribute to the pathological process of spontaneous abortion.

In summary, this study identifies RHOQ downregulation and its negative correlation with mature DC abundance as novel features associated with RSA. The transition from computational prediction to biological validation was achieved through immunohistochemical confirmation of RHOQ downregulation and mature DC increase in clinical tissues, followed by in vitro functional evidence showing that RHOQ silencing in endometrial cells promotes a DC-activating phenotype.

Collectively, our findings position RHOQ as a potential biomarker linked to DC dysregulation in RSA. To further elucidate its in vivo role and mechanistic basis, future work will focus on:To comprehensively characterize decidual immune dysregulation in RSA patients, we will expand our cohort to include a larger, well-phenotyped set of RSA patients and matched gestational-age-matched healthy controls. Subsequent single-cell RNA sequencing (scRNA-seq) of decidual immune cells will enable high-resolution comparative profiling—quantifying compositional shifts across major and rare immune subsets (e.g., NK cell subsets, macrophage polarization states, Treg/Th17 balance, dendritic cell subtypes, and γδ T cells) and inferring functional alterations through differential gene expression, pathway enrichment, ligand–receptor interaction analysis, and inferred cellular activation states; establishing an RHOQ-knockout mouse pregnancy model to assess its impact on endometrial receptivity and immune microenvironment dynamics; performing transcriptome sequencing of RHOQ-deficient models to identify downstream targets and pathways; and testing the involvement of RHOQ in GTPase-mediated signaling through correlation studies with key effectors (e.g., STAT3, ROCK) and functional rescue experiments, aiming to clarify its molecular role in immune tolerance and pregnancy maintenance.

Declarations

Competing interests

The authors have no financial conflicts of interest.

Ethics approval

This research was approved by the Institutional Review Board of the Fifth Affiliated Hospital of Guangzhou Medical University (approval no. KY01-2021-09-08).All human-related procedures were performed in accordance with the Declaration of Helsinki .

Informed consent

All participants provided written informed consent before the study.

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Author Contribution

Conceptualization: YYQ; Data curation: YYQ, YKM; Formal analysis: YMH, HJ; Investigation: QYY, YMH, HLW; Methodology: YYQ; Software: YYQ, YKM ; Supervision: HJ; Validation: HYM; Visualization: YYQ;Writing–original draft: YYQ.

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Data Availability

The datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus (GEO) repository, under the following accession numbers: GSE58144, GSE165004, GSE92324, GSE76862. All data are publicly accessible at https://www.ncbi.nlm.nih.gov/geo/.

Code availability

No datasets were generated during the current study.

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Figures

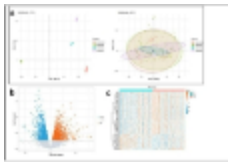


Figure 1

Genes that were differentially expressed between the RSA group and the normal pregnancy group were screened. 1a PCA plot after integration of the four databases. 1b Volcano plot of genes that are differentially expressed between RSA patients and patients with normal pregnancies. 1c Heatmap of the

top 100 genes that are differentially expressed between RSA patients and patients with normal pregnancies. *P<0.05

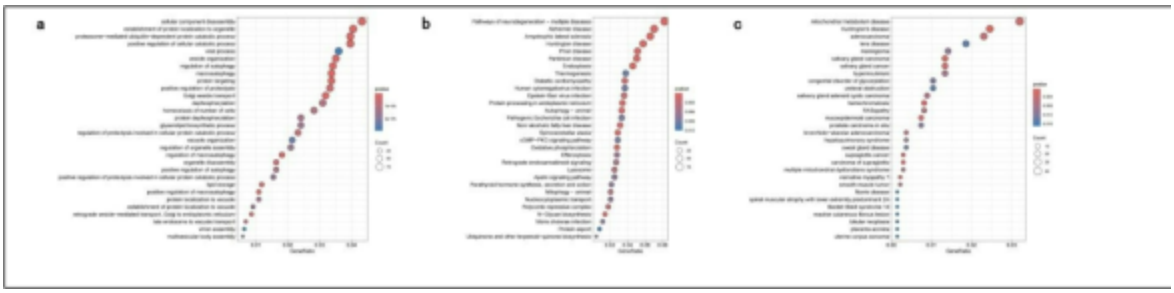


Figure 2

GO/DO/KEGG functional enrichment analysis of DEGs in DER patients and normal controls. 2a Plot of the top 30 enriched cellular component (CC) of GO terms of DEGs in DER and normal controls. 2b Plot of the top 30 enriched Disease of DO terms of DEGs in DER and normal controls. 2c Plot of the top 30 enriched signaling pathways of KEGG of DEGs in DER and normal controls. DEGs, differentially expressed genes. *P<0.05

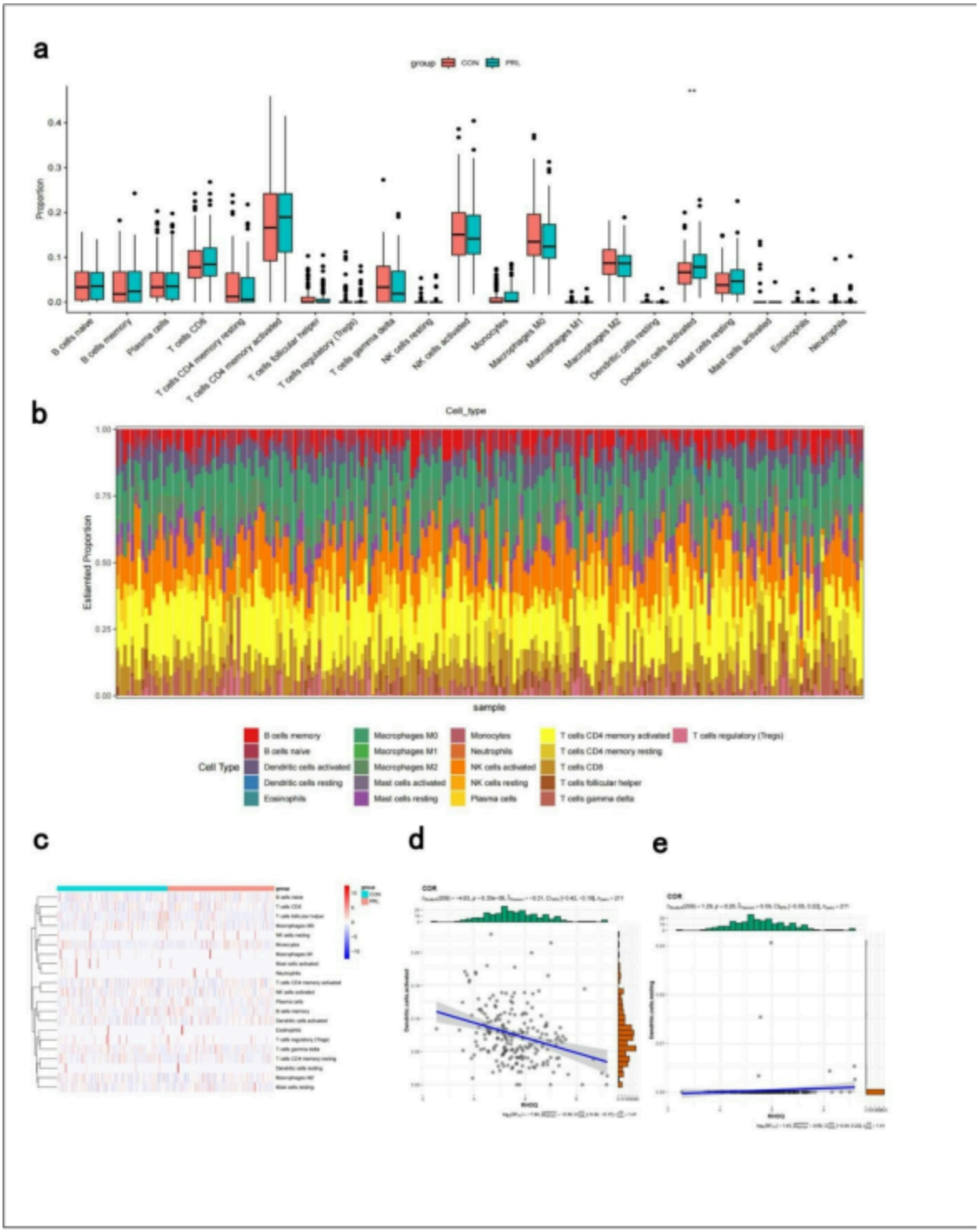


Figure 3

The proportion of mature dendritic cells was higher in the RSA group than that in the normal group and was negatively correlated with the expression of RHOQ. 3a Differential abundance of immune cells between the RSA group and the normal pregnancy group. 3b Hot spot map of the immune cell numbers of each sample; 3c Hot spot map of the immune cell numbers of the RSA group and the normal pregnancy group. 3d Pearson correlation analysis of immature DC cell numbers and RHOQ expression. *P<0.05

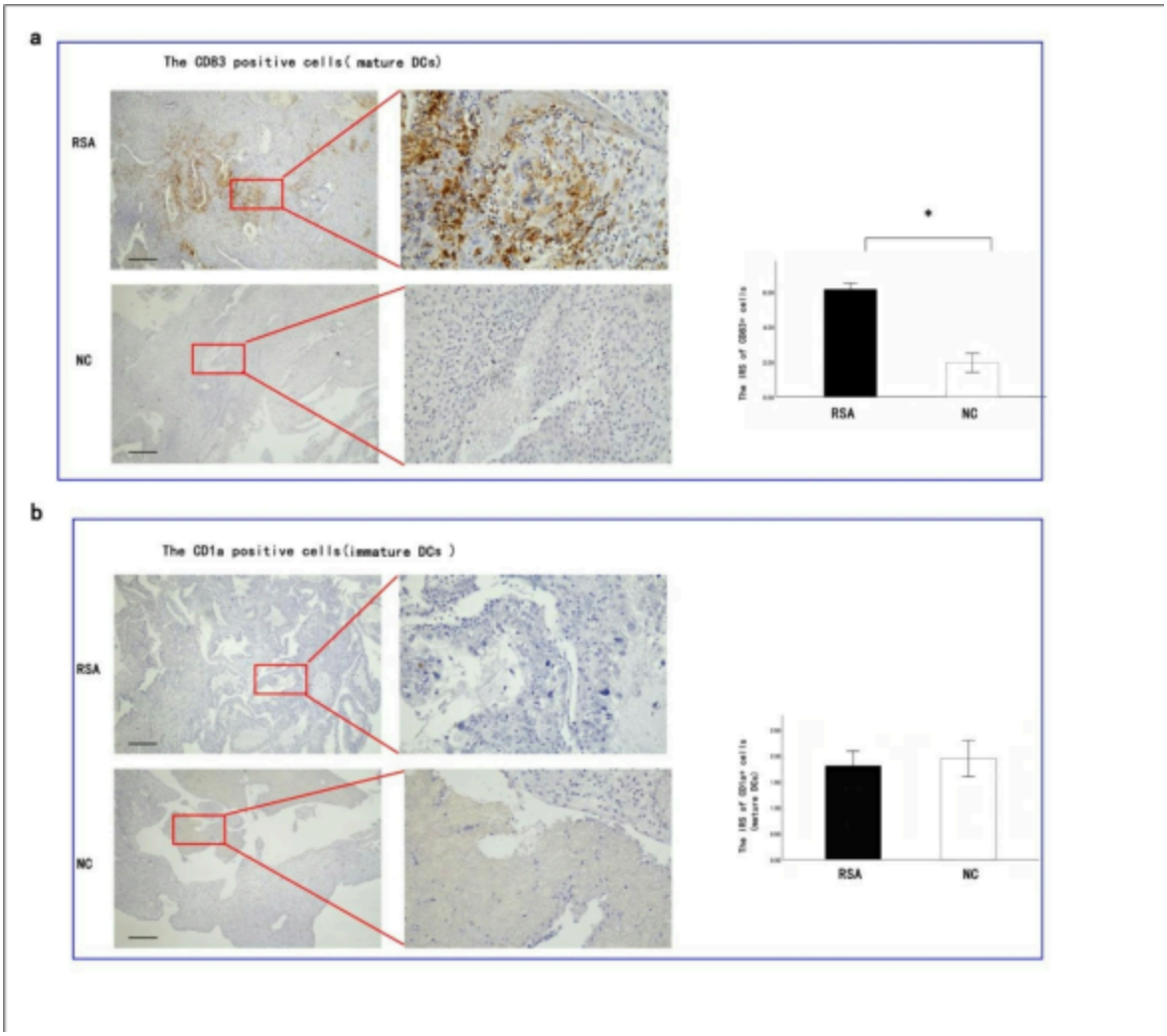


Figure 4

There were more mature dendritic cells in the decidual tissue of RSA group(n=40) than in that of the normal pregnancy group(n=20). 4a IRSs of CD83+ cells(mature DCs) in the RSA group and normal pregnancy group. 4b IRSs of CD1a+ cells(immature DCs) in the RSA group and normal pregnancy group.IRS scores were calculated by multiplying the score for the percentage of positively stained cells by the score for the staining intensity. *P<0.05, scale bar = 200 μm.

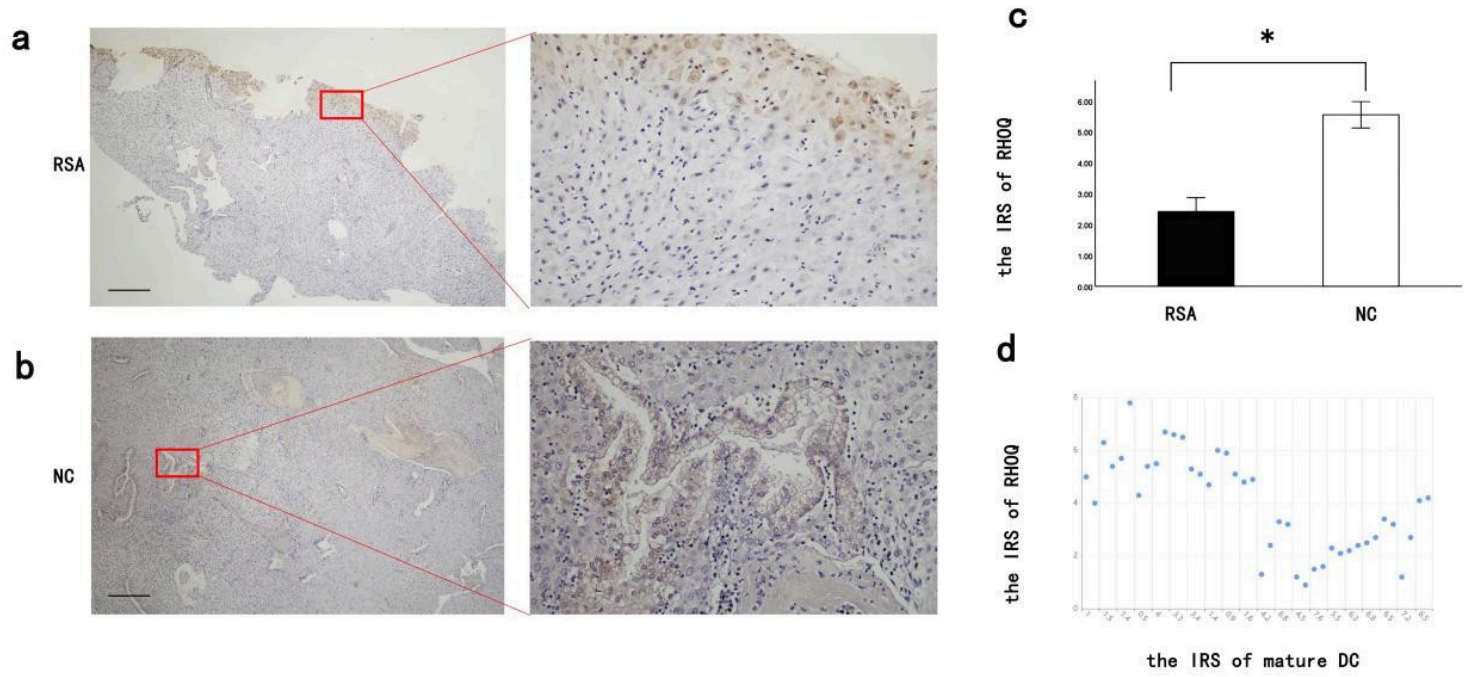


Figure 5

Analysis of RHOQ expression intensity in the RSA group and normal pregnancy group. 5a, 5b and 5c Immunohistochemical staining and expression intensity analysis of RHOQ in the RSA group and normal pregnancy group. 5d Correlation analysis of RHOQ expression intensity in mature DC cells in the RSA group and normal pregnancy group. *P<0.05, scale bar = 200 μm.

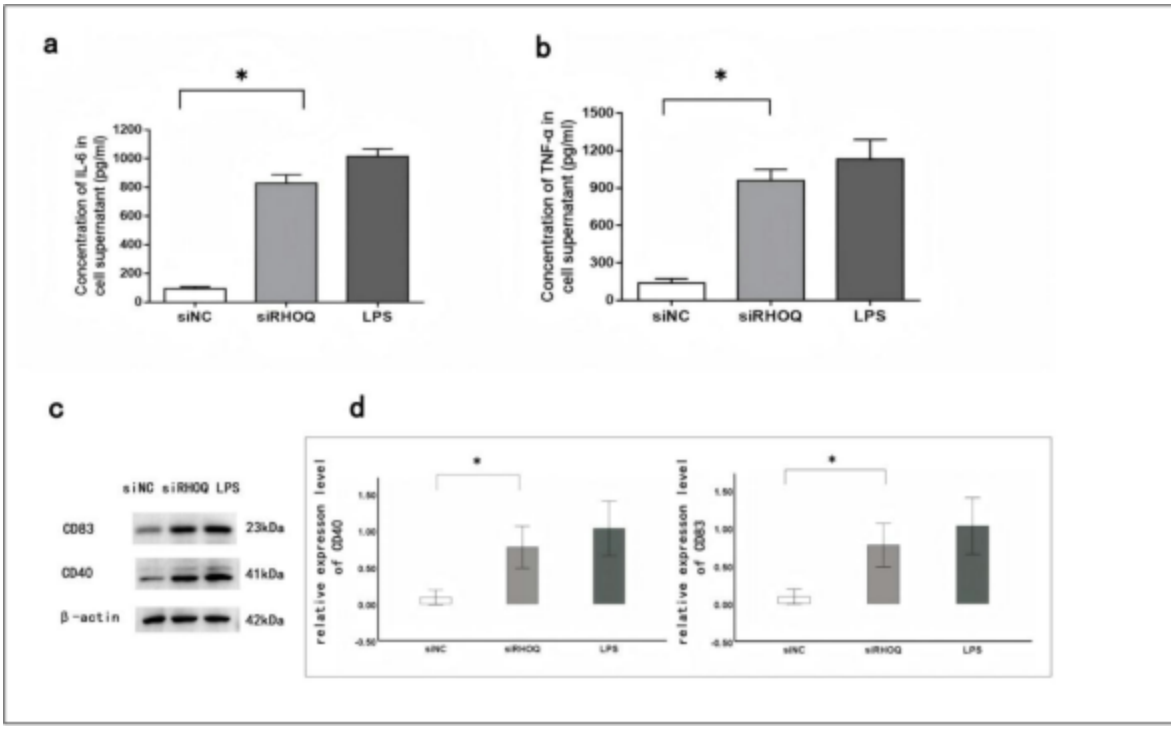


Figure 6

IL-6, TNF-α, CD40 and CD83 expression in the si-NC, si-RHOQ and LPS groups. 6a and b show IL-6 and TNF-α expression levels in the supernatants from three groups determined by ELISA. 6c and d show CD40 and CD83 protein expression levels among the three groups determined by WB. Experiments were conducted with 3 independent biological replicates per group. *P<0.05.

Supplementary Files

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

- Ethicalapprovaldocument.pdf
- SupplementaryTable6.pdf
- SupplementaryTable5.pdf