G-CSF: A Vehicle for Communication Between Trophoblasts and Macrophages, Which May Cause Problems in Recurrent Spontaneous Abortion

Peng Gao
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Haiyi Liu
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Ying Zha
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Lijie Wei
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Xuan Zhou
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Shenglan Zhu
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Huiting Zhang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Xuan Gao
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Yi Jiang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Yuting Chen
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Jiaqi Li
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Jingyi Zhang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Jun Yu
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Shaoshuai Wang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Ling Feng (✉ fltj007@163.com)
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology

Research

Keywords: Recurrent spontaneous abortion, Trophoblast, Macrophage, G-CSF, Inflammation

Posted Date: September 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-903181/v1

Version of Record: A version of this preprint was published at Placenta on March 1st, 2022. See the published version at https://doi.org/10.1016/j.placenta.2022.03.125.

Abstract

Background: The etiology of about half of patients with recurrent spontaneous abortion (RSA) remains unclear. Imbalance of the immune inflammatory response at the mother-foetal interface may be one of the keys to the onset of RSA. Granulocyte-colony stimulating factor (G-CSF) is thought to have a protective effect on pregnancy and its absence may lead to pregnancy failure. However, the evidence of the described effects of G-CSF is scant. This study aimed at investigating whether the loss of G-CSF induced RSA by affecting cell communication at the maternal-foetal interface.

Results: It was found that G-CSF was mainly expressed in villus rather than decidua and expression in RSA tissues was lower than that in normal tissues. Further, the down-regulation of G-CSF in trophoblasts resulted to a decrease in cell activity. Trophoblast-derived exosomes inhibited macrophage activation, while G-CSF free exosomes did not. Intraperitoneal injection of G-CSF improved the pregnancy outcome in RSA mice and the expression of G-CSF as well as its receptor at the mother-foetal interface were also changed.

Conclusion: The expression of G-CSF was found to be decreased in villi of patients with RSA. It was evident that the absence of G-CSF weakens the immune suppression of trophoblasts against macrophages and the function of trophoblasts is also impaired. Therefore, this may be a key factor in the occurrence of RSA. Further, G-CSF decreases the rate of abortion in RSA mice and may provide some assistance in the treatment of patients with RSA.

Background

Recurrent spontaneous abortion (RSA) is defined as two or more consecutive spontaneous abortions with the same sexual partner, with an incidence of about between 5 and 10% [1–3]. Among the reported incidences, approximately half of the cases remain unexplained [4, 5]. Our previous studies suggested that communication between trophoblasts and macrophages was deviated at the maternal-foetal interface of patients with RSA. Further, it was reported that the overexpression of NLRP3 inflammasomes triggered abnormal inflammatory response which destroyed the balance between pro-inflammatory and anti-inflammatory states at the maternal-foetal interface [6].

Proper communication between foetal trophoblast and immune cells present at the maternal-foetal interface including macrophages is essential for a successful pregnancy. Trophoblasts exhibit important roles in early embryo implantation and pregnancy. They invade the endometrium to establish maternal-foetal connection and promote the formation of maternal immune tolerance to the foetus [7]. Inadequate migration of trophoblasts usually leads to failure of maternal-foetal linkage and RSA [8, 9]. Meanwhile, increasing evidences support that the polarization of decidual macrophages is influenced by trophoblasts [10–13]. These decidual macrophages have considerable plasticity and can effectively respond to environmental signals. Furthermore, their phenotypes can be roughly divided into pro-inflammatory M1 type and anti-inflammatory M2 type. It has been reported that decidual macrophages are close to the M2

type and may rapidly transform into M1 type with the progress of pregnancy and environmental changes [11].

Granulocyte-colony stimulating factor (G-CSF) is a type of secretory cytokine closely related to cell proliferation, invasion and differentiation. It can be expressed in trophoblasts and endometrium. G-CSF has a positive effect on trophoblast growth, embryo implantation and placental metabolism [14, 15]. The function of G-CSF is mainly realized by binding to the G-CSF receptor (G-CSFR) on the surface of target cells and G-CSFR exists in various types of myeloproliferative tissues and cells, such as monocytes macrophages [16, 17]. Therefore, G-CSF may be one of the media for the communication between trophoblasts and decidual macrophages. Previous studies in animal models have shown that the loss of G-CSF may induce early abortion [16]. Some research reports have shown that *in vitro* injection of human recombinant G-CSF to patients with RSA may improve the pregnancy outcome and the efficacy is even better than that of clinical conventionally used drugs such as low molecular weight heparin and immunoglobulin, while others hold the opposite view [17–19]. It is hence evident that this problem requires further study.

In the last century, it was discovered that trophoblast vesicles might be shed from the placenta into the mother's peripheral circulation and could be isolated from the mother's peripheral blood as early as at 6 weeks of gestation [20, 21]. Recently, exosomes, a clearer concept than "vesicles", have gained increasing attention. Exosomes are membrane-bound vesicles, which exist in almost all biological fluids. Exosomes are rich in protein, RNA and have a strong function in information exchange between cells [22].

Trophoblast-derived exosomes are thought to have ability to regulate cytokine profile of recipient cells, such as increased IL-2 expression and decreased CD3 expression in the T cells. Literatures support that during implantation and the initial trimester, trophoblast-derived exosomes are capable of recruiting and promoting differentiation of monocytes into a trophoblast-supportive tissue macrophages. This in turn secrete cytokines and chemokines required for trophoblast growth and survival [23]. As a soluble cytokine, G-CSF can be released extracellularly or transported by extracellular vesicles such as exosomes to participate in the activation of cell signalling pathways in a paracrine form [24]. Therefore, the purpose of this study was to evaluate whether G-CSF carried by exosomes is involved in the trophoblast-macrophage dialogue and whether the absence of G-CSF is important factor leading to the occurrence of RSA.

Results

Expression of G-CSF and its receptor at maternal-foetal interface in early pregnancy

The presence of G-CSF and G-CSFR in villous and decidual tissues was detected using RTPCR, western blot and immunohistochemistry. It was found that G-CSF was mainly expressed in villi and the expression in RSA villi was significantly lower than that in normal villi. On the other hand, the G-CSFR showed an

opposite trend and its expression in RSA decidua was slightly higher than that in normal decidua (Fig. 1 and Additional file 1: Fig. S1).

Effect of G-CSF on migration and proliferation of trophoblasts

In this study, three siRNAs targeting G-CSF were constructed and the RTPCR detection results showed that all the three siRNAs had silencing effects. Further, the most effective siRNA was selected for subsequent experiments. Forty-eight hours after siRNA transfection, the cells were assayed by transwell and CCK8 tests. It was found that the absence of G-CSF significantly reduced the migration and proliferation of trophoblasts (Fig. 2 and Additional file 2: Fig. S2).

Role of G-CSF and exosomes in trophoblasts regulation on macrophages

The supernatant of trophoblasts after siRNA transfection or not were collected, exosomes were extracted and added into macrophages medium at a concentration of 100 μg/mL [23]. Recombinant G-CSF was separately added into the macrophages medium at concentrations of 10, 50, 100, 200 and 500 ng/mL. It was found that G-CSF had the most anti-inflammatory effect on macrophages at a concentration of 100 ng/ml hence this concentration was used in subsequent experiments.

The morphology, size and specific markers of exosomes were identified through transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blot (Fig. 3A). The particle size of exosomes was 134.70 ± 48.20 nm. Since the amplification of trophoblast took about a week and the effect of siRNA was not permanent, the expression of G-CSF in cells after transfection for 7 days was tested and it was still suppressed. The detection of G-CSF in exosomes also showed the silencing effect of transfection (Fig. 3B and Additional file 3: Fig. S3).

Under the laser confocal microscope, it was found that the PKH26 labelled exosomes were clearly engulfed by macrophages and were surrounded the nucleus. At low magnification, it was seen that the exosomes derived from trophoblasts treated with G-CSF siRNA were more phagocytosed by macrophages than exosomes derived from normal trophoblasts (Fig. 3C). After phagocytosis of exosomes, the functional activity of macrophages also changed. The normal trophoblasts downregulated the expression of iNOS and TNF-α and upregulated the expression of CD206 as well as Arg1 in macrophages through exosomes.

NLRP3 inflammasome is a molecule thought to play a pro-inflammatory role at the maternal-foetal interface in patients with RSA [6]. In this study NLRP3 was also found to be inhibited by trophoblast cell-derived exosomes. Therefore, results of the current study evidently indicates that trophoblasts can induce the transformation of macrophages to anti-inflammatory M2 type through exosomes. In addition, trophoblast cell-derived exosomes also had inhibitory effects on the migration and proliferation of macrophages. The addition of exogenous G-CSF had the similar effects on the macrophages. However,

the described effects disappeared after silencing the G-CSF in the trophoblasts. (Fig. 3D and Additional file 4: Fig. S4). This suggests that G-CSF plays an important role in the process of trophoblasts regulating macrophages.

Application of G-CSF in RSA mouse model

Results of this study show that the abortion rate in the RSA group was higher (26.96%) compared with the Normal group (5.71%) which indicates the success of the studied model. Expectedly, the abortion rate was reduced to 10.25% after intraperitoneal injection of G-CSF. Further, a decreased expression of G-CSF was also observed in the placentas of RSA mice. The increase of G-CSF in the TREAT group evidently show that intraperitoneal injection of G-CSF played a role in the placentas. There was no significant difference in the expression of G-CSFR among the three groups. Further, it was found that the expression of NLRP3 inflammasome was increased in the placentas of RSA mice and was inhibited by exogenous G-CSF, which was in consistence with the results of cell experiments (Fig. 4 and Additional file 5: Fig. S5).

Discussion

Occurrence of RSA is closely related to the imbalance of pro-inflammatory and anti-inflammatory state, and the formation barriers of immune tolerance at the maternal-foetal interface. The disorder of trophoblast-macrophage dialogue is likely to be a crucial part to induce the imbalance [10, 25, 26]. Previous studies have reported that G-CSF plays an important role in establishing immune tolerance between host tissue and donor-derived immune cells [27]. Therefore, the current aimed to investigate whether G-CSF plays the same role at the maternal-foetal interface.

First, tissue samples from the patients were examined and the results showed that G-CSF was mainly expressed in villous tissues and was lower in RSA samples than in the normal tissues. This was an initial suggestion of the potential association between G-CSF and RSA. On the contrary, G-CSFR was more expressed in decidua tissues and the expression in RSA decidua was slightly higher than that in the normal decidua. It was hence hypothesized that the increased expression of G-CSFR in the RSA decidua may be a compensatory response to the decreased G-CSF. That the migration and proliferation of trophoblasts decreased after down-regulation of G-CSF is a suggestion that G-CSF is important in the growth of trophoblasts. Therefore, the decline in G-CSF levels and the consequent impairment of trophoblast function may be the key factors in the induction of RSA.

In the pathogenesis of RSA, there is usually more than one abnormal link and the role of G-CSF at the maternal-foetal interface is not only to regulate the function of trophoblast cells. To investigate the role of macrophages in the pathogenesis of RSA, macrophages were co-cultured with trophoblast-derived exosomes. As expected, the exosomes without G-CSF did not inhibit macrophage activity, leading to more M1-type differentiation of macrophages and increased NLRP3 inflammasomes. The findings of this study was in consonance with our previous studies which have reported the association between the overexpression of NLRP3 inflammasomes and RSA [6, 28]. Therefore, it is suggested that the loss of G-CSF, on the one hand, disrupts the function of trophoblasts whereas on the other hand, interferes with the

pro-inflammatory and anti-inflammatory balance at the mother-foetal interface through macrophages. This then leads to impaired trophoblasts function [7, 29] and ultimately induces RSA.

As previously noted, the jury is still out on the efficacy of G-CSF in treating RSA and there is need for more evidence. Several studies have shown that G-CSF improves endometrial thickness, ovarian follicular function and oocyte quality, which may facilitate embryo implantation [30–32]. In the present study, intraperitoneal injection of G-CSF into RSA mice showed a significant reduction in abortion rate. Further, it was evident that G-CSF inhibited overexpression of NLRP3 inflammasomes at the maternal-foetal interface. Therefore, it is expected that G-CSF may improve pregnancy success by improving the inflammatory state of the mother-foetal interface and the activity of trophoblasts. However, there is still need for more research studies to provide more informed basis for the reported findings.

Conclusion

In conclusion, this study found that G-CSF was mainly expressed in villi during early pregnancy and the expression of G-CSF was decreased in villi of patients with RSA. The absence of G-CSF weakens the immune suppression of trophoblasts against macrophages and the function of trophoblasts is impaired, which may be a key factor in the occurrence of RSA. In addition, G-CSF decreased the rate of abortion in RSA mice and might provide some assistance in the treatment of patients with RSA.

Materials And Methods

Tissue samples

The study population was obtained from Tongji Hospital, Huazhong University of Science and Technology, China. The participants included were 11 women with an uncomplicated first-term pregnancy and 10 pregnant women diagnosed with RSA. The inclusion criteria for both groups were: The couples and embryos had normal chromosomes and no family genetic history, absence of anatomical deformity of reproductive organs, normal endocrine profile, normal coagulation and autoimmune function as well as no vaginal infections. No women received any treatment that affected the results of these examinations in the past 3 months before being enrolled in the study. The diagnostic criteria for RSA was two or more consecutive spontaneous abortions with the same sexual partner. There was no statistical difference in ages, gestational ages and menstrual cycle between the two groups. The protocol was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (2020-S150) and informed consent of all women was obtained. Villous and decidual tissues were collected during uterine curettage. The collected samples were washed immediately with normal saline and divided into two parts: one was fixed with polyformaldehyde for paraffin embedding and the other was stored at -80 ℃.

Cell culture

The trophoblast cell line HTR-8/SVneo was cultured in complete Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37°C. The macrophage cell line RAW264.7 was cultured in DMEM with 10% FBS, but no antibiotics, which might cause macrophage polarization. The cell was also placed in the cell incubator at 37 ℃ with 5% CO_2 in humidified air.

siRNA transfection

The HTR-8/SVneo Cells were seeded in a six-well plate with a density of between 40 and 60% for transfection. Transfection was performed using lipo3000 reagent (Invitrogen) in OPTI-MEM medium (Gibco) and siRNA (RiboBio), targeting the coding region of G-CSF (50 nM) and negative control siRNA (50 nM), according to the manufacturer's instructions. After 48 to 72 h of culture, the cells were collected for detection.

Exosomes isolation

The HTR-8/SVneo cells were planted in 10 cm petri dishes and cultured. The cells were then expanded in large quantities and culture medium was collected. The medium used to extract exosome was supercentrifuged overnight to remove its own exosome before cell culture. The medium was centrifuged at 300 g for 10 min, 2000 g for 30 min and 12000 g for 45 min to remove whole cells and debris. The resulting supernatant was passed through a 0.22 μm sterile filter and then ultra-centrifuged at 120000 g for 2 h. The exosome-containing pellets were washed in PBS, and ultra-centrifuged again at 120000 g for 2 h [33]. The final precipitates were dissolved in PBS and stored at − 80°C. The size and morphology of exosomes were identified through TEM and nanoparticle tracking analysis NTA.

Macrophage phagocytosis assay

The exosomes were incubated with PKH26 dye (Sigma) and the staining was stopped by serum, followed by ultra-centrifuged at 120000 g for 2 h. The obtained exosomes were added to the RAW264.7 culture medium at a concentration of 10 μg/mL. After 24 h of culture, the macrophages were fixed with paraformaldehyde, stained with DAPI, sealed with anti-fluorescence quenching agent and observed under fluorescence microscope as well as laser scanning confocal microscope.

Animals

Female CBA/J, male DBA/2J and male BALB/c mice (6 weeks old) were acquired from Wuhan SLB Biotech CO., LTD for the animal studies. All the studies were performed in the Laboratory Animal Centre, Tongji Hospital. The experimental protocols of this study were approved by the Animal Ethics Committee of Tongji Hospital (TJH-202009005). Mice were reared under controlled temperature (21 ℃ -22 ℃), humidity, and 12 h of a light/dark cycle with water and food ad libitum for 2 weeks. Female and male mice were mated in the ration of 2:1 at 20:00 hrs and checked at 6:00 hrs the next day for vaginal plugs. The presence of a vaginal plug was designated as day 0.5 of pregnancy. Pregnant mice received daily intraperitoneal injections of recombinant G-CSF or saline from day 4.5 to 13.5. CBA/J females that mated with BALB/c were considered as normal group and received 200 μl saline (n = 6), and CBA/J females that

mated with DBA/2 were randomized and divided into the following groups: RSA group received 200 μl saline (n = 6) and TREAT group received 200 μl of 50 μg/kg recombinant G-CSF [34–36]. Pregnant females were killed at day 14.5, the total number of resorbing and healthy embryos were recorded, the foetal and placenta weight were also determined and the placentas were fixed with polyformaldehyde for paraffin embedding. Abortion sites were identified by their small size accompanied by a necrotic, haemorrhagic appearance compared to normal embryos and placentas. The resorption rate (%) was calculated as the number of total abortion divided by the number of total implantation and multiplied by100.

Immunohistochemistry

Patients and mice paraffin sections were deparaffinised and rehydrated. Slides were then incubated in citric buffer for antigen retrieval. The sections were treated with 3% hydrogen peroxide to suppress endogenous peroxidase activity. After incubation with 10% donkey serum, primary rabbit antibodies (G-CSF, abcam, diluted 1:200; G-CSFR, GeneTex, diluted 1:200; NLRP3, abcam, diluted 1:200) were applied overnight at 4°C. The second goat-anti-rabbit antibody was incubated after washing with PBS for three times. Diaminobenzidine was used as a chromogen and a light counterstaining was performed with haematoxylin. After dehydration and drying, the sections were sealed with neutral resin and observed under an optical microscope. The images were analysed semi-quantitatively using imageJ software and MOD = intDen/Area.

RNA isolation and quantitative real-time polymerase chain reaction (RTPCR)

Total RNA was extracted from tissues or cells using TRIzol reagent. Two μg RNA of every sample was converted to cDNA through reverse transcription. Quantitative real-time PCR analysis of genes was done using primers described in an additional file (Additional file 6: Table S1) and detected using the CFX96TM real-time system. Gene expression data was normalized to the mRNA levels of housekeeping gene GAPDH.

Western blot

Total proteins from tissues or cells were lysed using RIPA buffer. Twenty μg of proteins was loaded on a 10% Bis-Tris SDS gel and run for approximately 2 h at 120 V and then transferred to the PVDF membranes. The membranes were then blocked in 5% BSA and immunoblotted with primary antibodies (G-CSF, abcam, diluted 1:1000; G-CSFR, GeneTex, diluted 1:1000; CD9, abcam, diluted 1:1000; TSG101, abcam, diluted 1:1000; calnexin, abcam, diluted 1:1000; NLRP3, abcam, diluted 1:1000; GAPDH, abcam, diluted 1:1000) overnight at 4°C. Membranes were washed with TBST and incubated in the goat anti-rabbit or anti-mouse secondary antibodies (Servicebio, diluted 1:5000) for 1 h at room temperature. After three washes with TBST, the membranes were coloured by an ECL system. The images were then analysed semi-quantitatively using imageJ statistical software. Lastly, the data on protein expression were normalized to the levels of GAPDH.

Migration assay

Cell migration was evaluated using a Transwell chamber. Cells suspended in serum-free media were seeded in the upper chambers at a density of 2×10^4 cells per well. Complete DMEM (500 μL) with 20% FBS was added to the lower chambers. After 24 hours of culturing, the cells in the upper chamber were removed with a cotton swab and the cells on the lower surface were fixed and stained with purple crystal. Excised and mounted filter membranes were photographed using an optical microscope and cells in four random fields per sample were counted and recorded.

Proliferation assay

Cell proliferation was determined through CCK8 assay. Cells were plated in 96-well plates at a density of 4×10^3 cells per well. The CCK8 mixture was prepared according to DMEM:CCK8 = 9:1 and added to each well at 0 h, 12 h, 24 h, 48 h and 72 h after cell intervention. Absorbance was measured at 450 nm using an enzyme-labelled instrument after 2 h of culture.

Statistical analysis

SPSS version 22.0 software was used for data analysis. Results were presented as mean ± SEM. One-way analysis of variance (ANOVA) was used to assess statistical significance among various experiment groups followed by Bonferroni post hoc analysis. Statistically significant difference were set at $p < 0.05$.

Abbreviations

RSA: Recurrent spontaneous abortion

G-CSF: Granulocyte-colony stimulating factor

G-CSFR: Granulocyte-colony stimulating factor receptor

TEM: Transmission electron microscopy

NTA: Nanoparticle tracking analysis

Declarations

Acknowledgments

Not applicable.

Authors' contributions

LF, HL and PG conceived and designed the experiments; HZ, YJ, YC and SZ performed the experiments; PG, LW, YZ, XZ and JZ analysed the data and interpreted the results of the experiments; PG, JL and XG drafted the manuscript; LF, JY and SW reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

Funding

This project was funded by the National Key Research and Development Program of China and the Health Commission of Hubei Province scientific research project (No.2018YFC1002903 and No.WJ2021M129)

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (2020-S150) and the Animal Ethics Committee of Tongji Hospital (TJH-202009005).

Consent for publication

All authors consent for publication.

Competing interests

The authors declare no conflict of interest.

References

1. Pfeifer S, Goldberg J, Lobo R, Thomas M, Widra E, Licht M, et al. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil Steril. 2013;99(1):63.
2. Garrido-Gimenez C, Alijotas-Reig J. Recurrent miscarriage: causes, evaluation and management. Postgrad Med J. 2015;91(1073):151-62.
3. Zhu XX, Yin XQ, Hei GZ, Wei R, Guo Q, Zhao L, et al. Increased mir-6875-5p inhibits plasmacytoid dendritic cell differentiation via the stat3/e2-2 pathway in recurrent spontaneous abortion. Mol Hum Reprod. 2021;
4. Sugiura-Ogasawara M, Ozaki Y, Suzumori N. Management of recurrent miscarriage. J Obstet Gynaecol Re. 2014;40(5):1174-9.
5. Grandone E, Tiscia GL, Mastroianno M, Larciprete G, Kovac M, Tamborini Permunian E, et al. Findings from a multicentre, observational study on reproductive outcomes in women with unexplained recurrent pregnancy loss: the ottilia registry. Hum Reprod. 2021;36(8):2083-90.
6. Gao P, Zha Y, Gong X, Qiao F, Liu H. The role of maternal–foetal interface inflammation mediated by NLRP3 inflammasome in the pathogenesis of recurrent spontaneous abortion. Placenta. 2020;101:221-9.

7. Ding J, Zhang Y, Cai X, Zhang Y, Yan S, Wang J, et al. Extracellular vesicles derived from m1 macrophages deliver mir-146a-5p and mir-146b-5p to suppress trophoblast migration and invasion by targeting traf6 in recurrent spontaneous abortion. Theranostics. 2021;11(12):5813-30.
8. Tian F, Qin C, Li X, Wu F, Liu X, Xu W, et al. Decreased stathmin-1 expression inhibits trophoblast proliferation and invasion and is associated with recurrent miscarriage. The American Journal of Pathology. 2015;185(10):2709-21.
9. Ding J, Cheng Y, Zhang Y, Liao S, Yin T, Yang J. The mir-27a-3p/usp25 axis participates in the pathogenesis of recurrent miscarriage by inhibiting trophoblast migration and invasion. J Cell Physiol. 2019;234(11):19951-63.
10. Aldo PB, Racicot K, Craviero V, Guller S, Romero R, Mor G. Trophoblast induces monocyte differentiation into cd14+/cd16+ macrophages. Am J Reprod Immunol. 2014;72(3):270-84.
11. Wang XQ, Zhou WJ, Hou XX, Fu Q, Li DJ. Trophoblast-derived cxcl16 induces m2 macrophage polarization that in turn inactivates nk cells at the maternal-fetal interface. Cell Mol Immunol. 2018;15(12):1038-46.
12. Wang S, Sun F, Han M, Liu Y, Zou Q, Wang F, et al. Trophoblast-derived hyaluronan promotes the regulatory phenotype of decidual macrophages. Reproduction. 2019;157(2):189-98.
13. Zhang YH, Aldo P, You Y, Ding J, Kaislasuo J, Petersen JF, et al. Trophoblast-secreted soluble-pd-l1 modulates macrophage polarization and function. J Leukocyte Biol. 2020;108(3):983-98.
14. Smith A, Witte E, McGee D, Knott J, Narang K, Racicot K. Cortisol inhibits csf2 and csf3 via dna methylation and inhibits invasion in first-trimester trophoblast cells. Am J Reprod Immunol. 2017;78(5):1-14.
15. Furmento VA, Marino J, Blank VC, Roguin LP. The granulocyte colony-stimulating factor (g-csf) upregulates metalloproteinase-2 and vegf through pi3k/akt and erk1/2 activation in human trophoblast swan 71 cells. Placenta. 2014;35(11):937-46.
16. Scarpellini F, Klinger FG, Rossi G, Sbracia M. Immunohistochemical study on the expression of g-csf, g-csfr, vegf, vegfr-1, foxp3 in first trimester trophoblast of recurrent pregnancy loss in pregnancies treated with g-csf and controls. Int J Mol Sci. 2020;21(1):285.
17. Würfel W. Treatment with granulocyte colony-stimulating factor in patients with repetitive implantation failures and/or recurrent spontaneous abortions. J Reprod Immunol. 2015;108:123-35.
18. Eapen A, Joing M, Kwon P, Tong J, Maneta E, De Santo C, et al. Recombinant human granulocyte-colony stimulating factor in women with unexplained recurrent pregnancy losses: a randomized clinical trial. Hum Reprod. 2019;34(3):424-32.
19. Eftekhar M, Naghshineh E, Khani P. Role of granulocyte colony-stimulating factor in human reproduction. J Res Med Sci. 2018;23:7.
20. Covone AE, Mutton D, Johnson PM, Adinolfi M. Trophoblast cells in peripheral blood from pregnant women. Lancet. 1984;2(8407):841-3.
21. Johansen M, Redman CW, Wilkins T, Sargent IL. Trophoblast deportation in human pregnancy–its relevance for pre-eclampsia. Placenta. 1999;20(7):531-9.

22. Xiang YJ, Hou YY, Yan HL, Liu H, Ge YX, Chen N, et al. Mesenchymal stem cells-derived exosomes improve pregnancy outcome through inducing maternal tolerance to the allogeneic fetus in abortion-prone mating mouse. Kaohsiung J Med Sci. 2020;36(5):363-70.
23. Atay S, Gercel-Taylor C, Suttles J, Mor G, Taylor DD. Trophoblast-derived exosomes mediate monocyte recruitment and differentiation. Am J Reprod Immunol. 2011;65(1):65-77.
24. Rodrigues C, Serrano E, Patricio MI, Val MM, Albuquerque P, Fonseca J, et al. Stroma-derived il-6, g-csf and activin-a mediated dedifferentiation of lung carcinoma cells into cancer stem cells. Sci Rep. 2018;8(1):11573.
25. Di Nicuolo F, Specchia M, Trentavizi L, Pontecorvi A, Scambia G, Di Simone N. An emerging role of endometrial inflammasome in reproduction: new therapeutic approaches. Protein Peptide Lett. 2018;25(5):455.
26. Li Y, Zhang D, Xu L, Dong L, Zheng J, Lin Y, et al. Cell-cell contact with proinflammatory macrophages enhances the immunotherapeutic effect of mesenchymal stem cells in two abortion models. Cell Mol Immunol. 2019;16(12):908-20.
27. Wen Q, Kong Y, Zhao H, Zhang Y, Han T, Wang Y, et al. G-csf-induced macrophage polarization and mobilization may prevent acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transpl. 2019;54(9):1419-33.
28. D'Ippolito S, Tersigni C, Marana R, Di Nicuolo F, Gaglione R, Rossi ED, et al. Inflammosome in the human endometrium: further step in the evaluation of the "maternal side". Fertil Steril. 2016;105(1):111-8.
29. Ding J, Yang C, Zhang Y, Wang J, Zhang S, Guo D, et al. M2 macrophage-derived g-csf promotes trophoblasts emt, invasion and migration via activating pi3k/akt/erk1/2 pathway to mediate normal pregnancy. J Cell Mol Med. 2021;25(4):2136-47.
30. Gleicher N, Vidali A, Barad DH. Successful treatment of unresponsive thin endometrium. Fertil Steril. 2011;95(6):2113-23.
31. Salmassi A, Schmutzler AG, Huang L, Hedderich J, Jonat W, Mettler L. Detection of granulocyte colony-stimulating factor and its receptor in human follicular luteinized granulosa cells. Fertil Steril. 2004;81 Suppl 1:786-91.
32. Ledee N, Lombroso R, Lombardelli L, Selva J, Dubanchet S, Chaouat G, et al. Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. Hum Reprod. 2008;23(9):2001-9.
33. Rice GE, Scholz-Romero K, Sweeney E, Peiris H, Kobayashi M, Duncombe G, et al. The effect of glucose on the release and bioactivity of exosomes from first trimester trophoblast cells. J Clin Endocrinol Metab. 2015;100(10):E1280-8.
34. Li Y, Guo R, Wang L, Li S, Zhu Z, Tu P. G-csf administration results in thrombocytopenia by inhibiting the differentiation of hematopoietic progenitors into megakaryocytes. Biochem Pharmacol. 2019;169:113624.

35. Skaznik-Wikiel ME, Sharma RK, Selesniemi K, Lee HJ, Tilly JL, Falcone T. Granulocyte colony-stimulating factor in conjunction with vascular endothelial growth factor maintains primordial follicle numbers in transplanted mouse ovaries. Fertil Steril. 2011;95(4):1405-9.
36. Lee HJ, Choi CW, Kim EK, Kim HS, Kim BI, Choi JH. Granulocyte colony-stimulating factor reduces hyperoxia-induced alveolarization inhibition by increasing angiogenic factors. Neonatology. 2012;101(4):278-84.

Figures

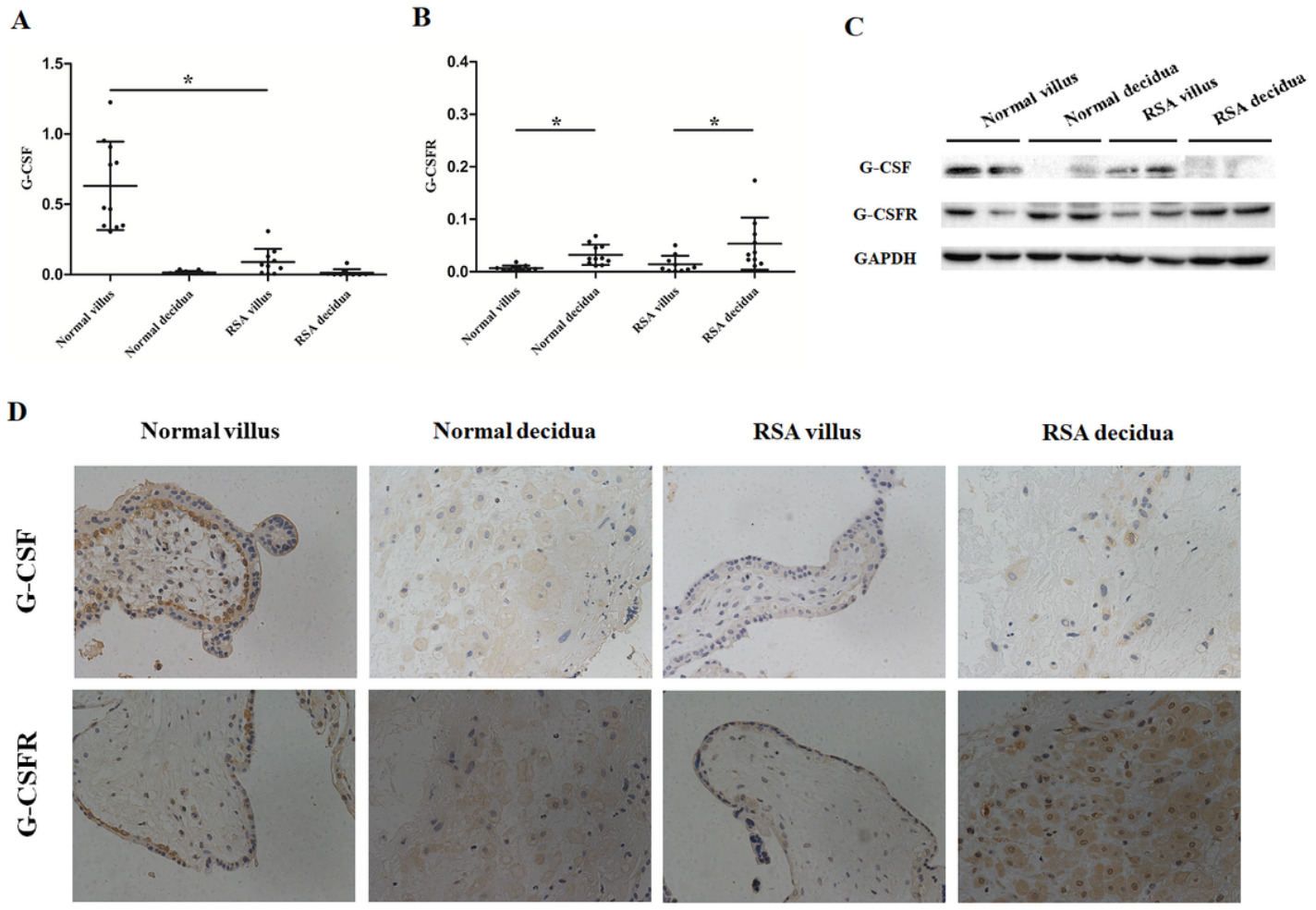

Figure 1

Expression of G-CSF and G-CSFR in villous and decidual tissues. Samples were detected by RTPCR (A, B, *: P < 0.05), western blot (C) and immunohistochemistry (D, original magnification × 200).

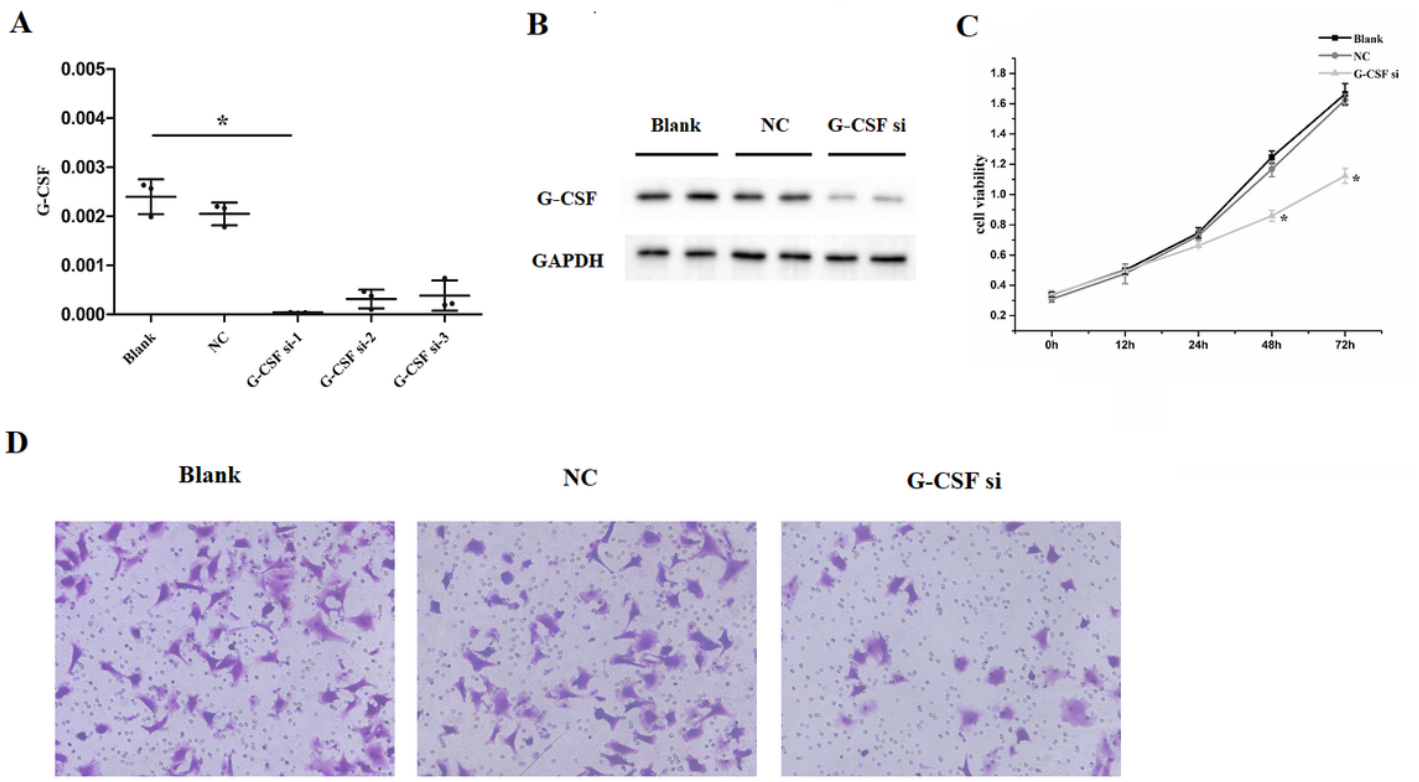

Figure 2

Effects of down-regulation of G-CSF on trophoblasts. (A) The silencing effect of three siRNAs against G-CSF was detected by RTPCR, *: $P < 0.05$. (B) The most effective siRNA was selected and verified by Western blot. (C) Proliferative capacity of trophoblasts was evaluated by CCK8 test, *: $P < 0.05$ vs Blank and NC. (D) Migratory capacity of trophoblasts was evaluated by transwell test, original magnification × 100. All data was from three independent experiments.

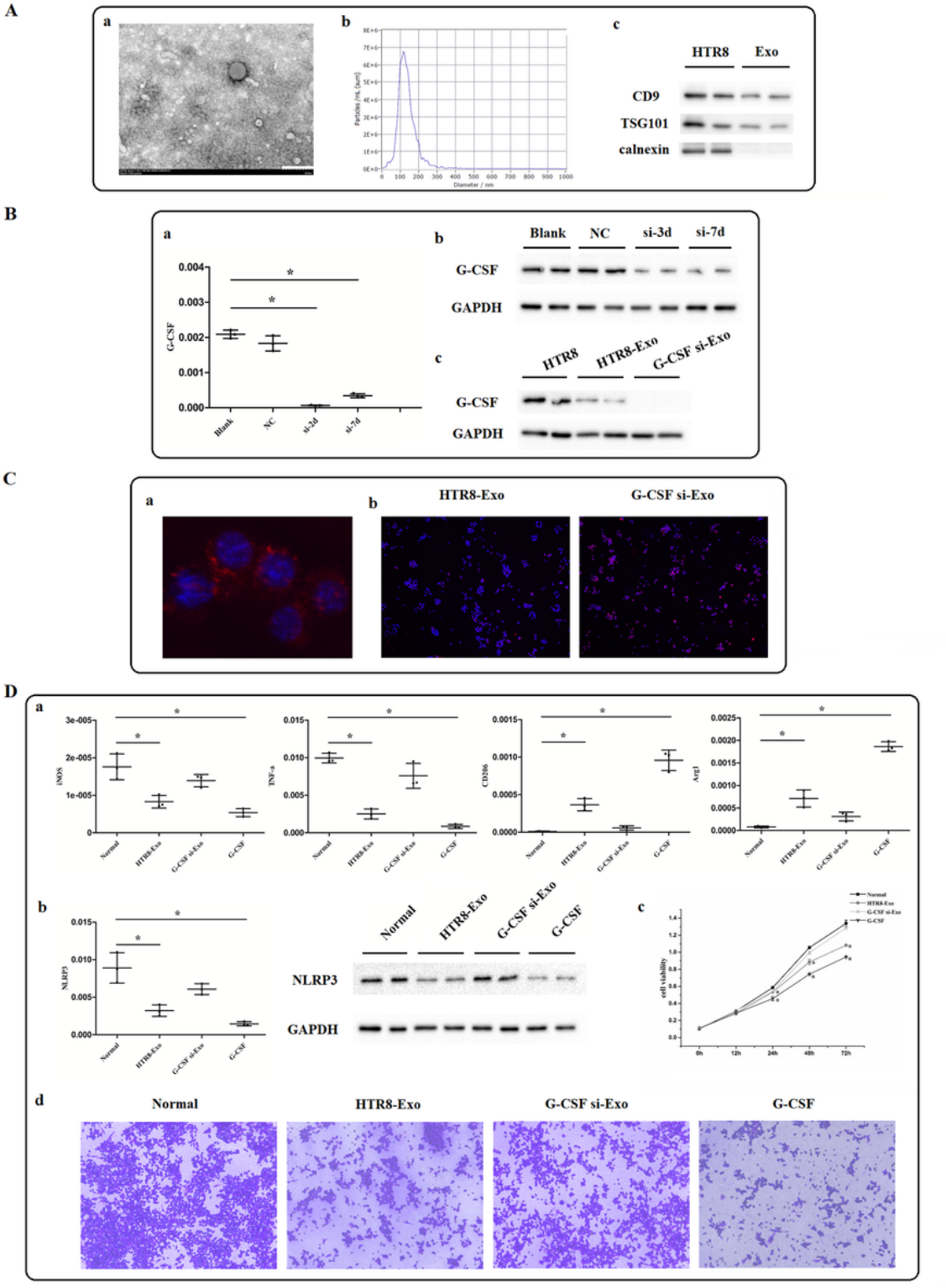

Figure 3

Role of G-CSF and Exosomes in Trophoblasts Regulation on Macrophages. (A) Exosomes derived from trophoblasts were identified by TEM (a, Scale bar: 200nm), NTA (b) and Western blot (c). (B) The expression of G-CSF in trophoblasts after siRNA transfection for 2-3 days and 7 days was detected by RTPCR (a, *: P<0.05) and western blot (b); the expression of G-CSF in exosomes derived from normal trophoblasts and trophoblasts transfected with siRNA was detected by Western blot (c). (C) Uptake of

exosomes by macrophages was observed by laser scanning confocal microscope (a, original magnification × 400 and zoom × 5) and fluorescence microscope (b, original magnification × 100). (D) Markers for macrophage type, iNOS, TNF-α, CD206 and Arg1, were detected by RTPCR (a, *: P<0.05); the expression of NLRP3 inflammasome in macrophages was detected by RTPCR and Western blot (b, *: P<0.05); proliferative capacity of macrophages was evaluated by CCK8 test (c, *: P<0.05); migratory capacity of macrophages was evaluated by transwell test (d, original magnification × 100). All data was from three independent experiments.

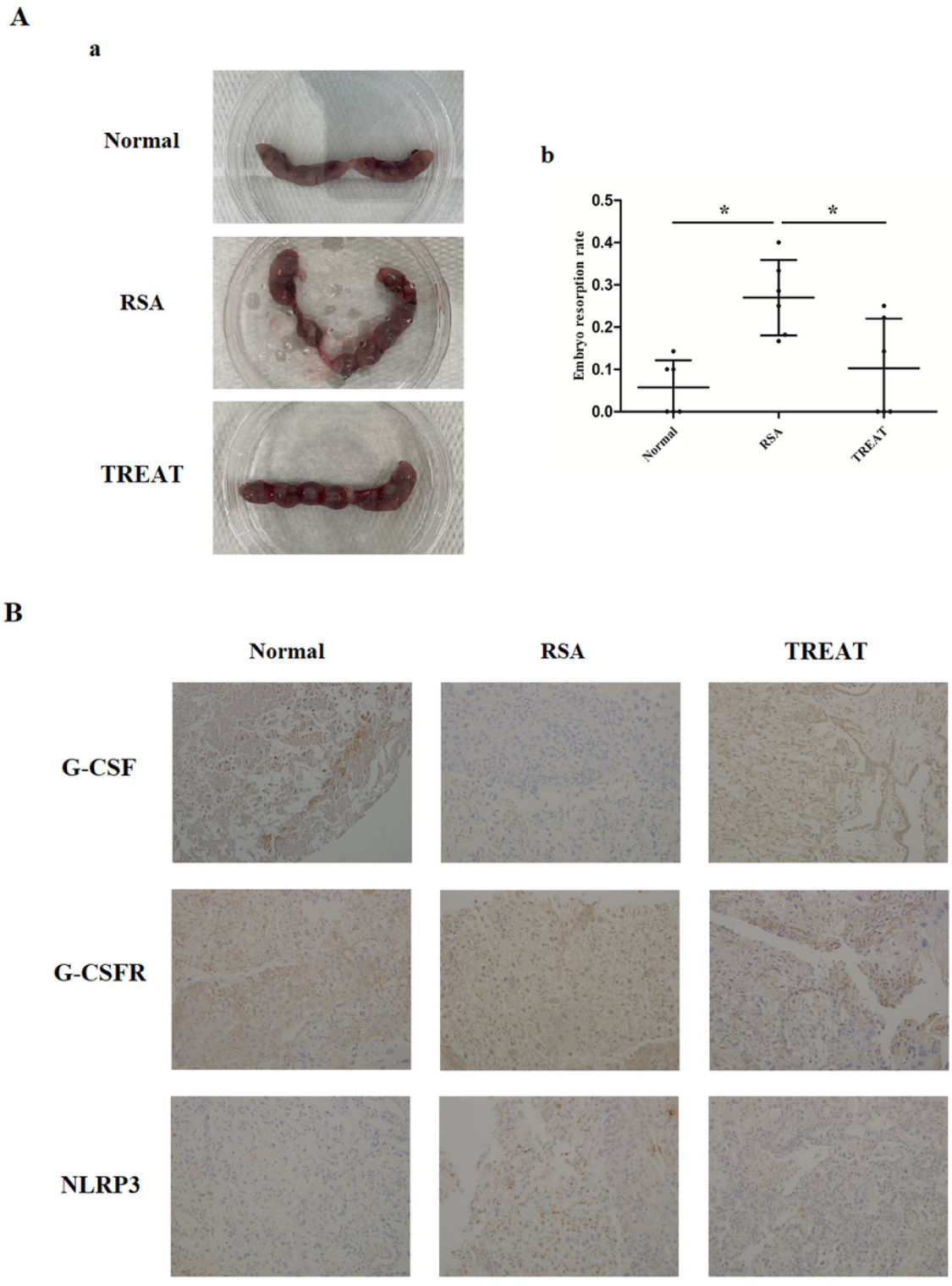

Figure 4

The pregnancy outcome of the mice model and the molecular expression at the maternal-foetal interface. (A) Representative pictures of uteri from three groups of mice (a) and embryo resorption rate of mice (b, *: $P < 0.05$). (B) Expression of G-CSF, G-CSFR and NLRP3 in placentas from three groups of mice, original magnification × 200.

Supplementary Files

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

- Additionalfile1.FigureS1.tif
- Additionalfile2.FigureS2.tif
- Additionalfile3.FigureS3.tif
- Additionalfile4.FigureS4.tif
- Additionalfile5.FigureS5.tif
- Additionalfile6.TableS1.docx