Molecular Human Reproduction, Vol.26, No.2, pp. 101–110, 2020
Advance Access Publication on January 3, 2020 doi:10.1093/molehr/gaz068

molecular human reproduction

ORIGINAL RESEARCH

Role of intrauterine administration of transfected peripheral blood mononuclear cells by GM-CSF on embryo implantation and pregnancy rate in mice

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Submitted on September 18, 2019; resubmitted on December 2, 2019; editorial decision on December 30, 2019

ABSTRACT: One of the effective treatments in women with recurrent implantation failure (RIF) is the use of immune cells to facilitate embryo implantation. Previous studies have shown that intrauterine transmission of peripheral blood mononuclear cells (PBMC) increased the embryo implantation rate. In this study using B6D2F1 (C57BL/6 × DBA2) mice, a fragment of the granulocyte macrophage colony-stimulating factor (*Gm-csf*) gene was cloned into an enhanced green fluorescent protein vector (pEGFP-N1) and then transfected into PBMC. The protein level of GM-CSF was evaluated in the transfected PBMC and untransfected PBMC by ELISA. Attachment of mouse embryos and the mRNA expression levels of leukemia inhibitory factor (*Lif*), vascular endothelial growth factor (*Vegf*), matrix metalloproteinase 9 (*Mmp9*), Gmcsf-receptor (*Gmcsf-r*) and interleukin 6 (*Il6*) *in vitro* were assessed by real-time PCR in endometrial cells. To determine the pregnancy rate and number of implantation sites *in vivo*, the mouse uterine horns were analyzed on Day 7.5 post coitum. A greater amount of GM-CSF was produced in PBMC transfected with recombinant vector (552 pg/mL) compared with the untransfected PBMC (57 pg/mL) and PBMC transfected with empty vector (34 pg/mL) ($P < 0.05$). The data showed that the embryo attachment rate and mRNA expression levels (*Vegf* [1.7-fold], *Mmp9* [1.4-fold], *Lif* [1.5-fold], *Gm-csf r* [1.6-fold] and *Il6* [1.2-fold]) in the *in vitro* study ($P < 0.01$), pregnancy rate ($P < 0.01$) and number of implantation sites ($P < 0.01$) in the *in vivo* investigation ($P < 0.05$) were increased in PBMC transfected with recombinant vector compared with the PBMC group. The study demonstrated that, in mice, endometrium immunotherapy with transfected PBMC that contained recombinant GM-CSF before embryo implantation was effective in improving embryo implantation and endometrial receptivity.

Key words: implantation / infertility / cytokines / granulocyte macrophage colony-stimulating factor / peripheral blood mononuclear cells / recurrent implantation failure

Introduction

Recurrent implantation failure (RIF) is one of the main concerns of patients who refer to infertility centers (Yakin *et al*., 2019). Embryo implantation, or placement of a blastocyst in the uterus, is an elementary stage in the reproductive process. Numerous researchers have reported that a poorly developed endometrium can lead to infertility. Steroid hormone supplements used to differentiate the endometrium in infertility patients have not been very effective (Egawa *et al*., 2002; Nakayama *et al*., 2002). Evidence has shown that immune cells located at the implantation site create a powerful inflammatory response that provides conditions for blastocyst adherence in the endometrium. Therefore, immune cell therapy may potentially be a good choice for the treatment of patients with RIF (Fujita *et al*., 1998; Hashii *et al*., 1998; Yakin *et al*., 2019).

Maternal peripheral blood mononuclear cells (PBMC) that consist of lymphocytes and monocytes interact with trophoblasts and create inflammatory responses. Numerous molecules, such as cytokines and chemokines secreted from lymphocytes, could be beneficial for successful blastocyst implantation in the uterus (Hashii *et al*., 1998; Ideta

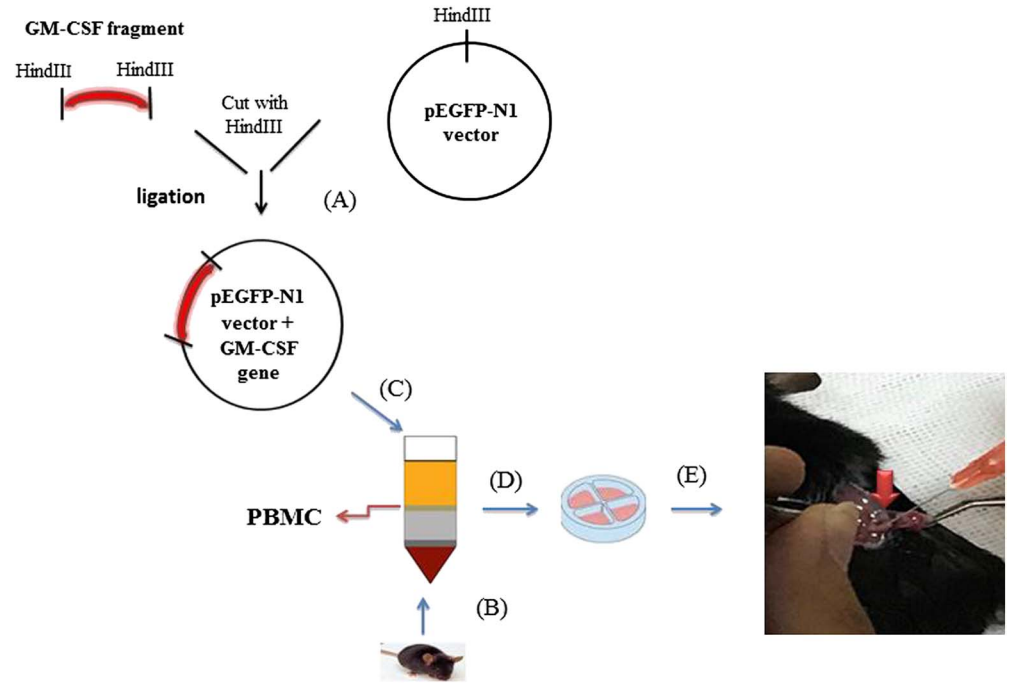

Figure 1 Summary of the experiment performed in B6D2F1 (C57BL/6 × DBA2) mice. (**A**) *Gm-csf* fragment was cloned into pEGFP-N1 vector. (**B**) Whole blood was isolated from a non-pregnant mouse and PBMC collected by gradient centrifugation. (**C**) PBMC cells were transfected by electroporation with a pEGFP-N1 expression vector that contained *Gm-csf* (pEGFP-N1-*Gm-csf*). (**D**) PBMC cultured in RPMI 1640 media. (**E**) Transfected PBMC was transferred into the uterine horn (arrow) of pregnant mice on post-coitum Day 1.5. Gm-csf: granulocyte-macrophage colony-stimulating factor, PBMC: peripheral blood mononuclear cell, pEGFP-N1: plasmid expressing green fluorescent protein-N1.

et al., 2010). Studies also demonstrated that incubation of endometrial epithelial cells with PBMC isolated from patients with RIF could increase the attachment of the embryo to the uterus (Fujita et al., 1998; Ideta et al., 2010; Kosaka et al., 2003; Lee et al., 2011; Madkour et al., 2016). The key role of PBMC is stimulation of cytokine and chemokine production, which is crucial for the establishment of pregnancy (Egawa et al., 2002; Yu et al., 2014). Some studies also did not show a significant difference in the live birth rates among PBMC-treated patients and untreated patients (Okitsu et al., 2011a; Yakin et al., 2019a). One of the cytokines produced at a relatively low rate by PBMC is granulocyte macrophage colony-stimulating factor (GM-CSF). GM-CSF stimulates the proliferation of multipotent progenitor cells, including macrophages and granulocytes. This cytokine plays a major role in the maintenance of embryo viability, development of pre-implantation embryo chemotaxis and synthesis of cytokines, phagocytosis and immune accessory molecules. In addition, up-regulation of the *GM-CSF* mRNA expression could promote augmentation of progesterone level and consequently prepare the uterus for embryo implantation (Rosendaal, 1975; Sjoblom et al., 1999; Robertson et al., 2000; Hamilton & Anderson, 2004; Dimitriadis et al., 2005; Chaouat et al., 2007; Yang et al., 2016). Researchers showed that the addition of GM-CSF to culture medium increased the human embryo implantation rate (Sjoblom et al., 1999). Also, the injection of recombinant GM-CSF in mice uteri could effectively promote the accumulation of immune cells and underlying inflammatory response in the uteri (Robertson et al., 2000). A barrier to the use of GM-CSF cytokine as an effective factor in implantation is its high cost (Puetz & Wurm, 2019). As mentioned, lymphocytes are the primary cells produced by GM-CSF. A probable mechanism for PBMC to impact implantation could be through the production of GM-CSF. However, GM-CSF is produced at low levels (Rosendaal, 1975; Hamilton & Anderson, 2004). Therefore, we propose a new procedure to increase embryo implantation. The objective of this study was to transfect murine PBMC with a green fluorescent protein expression vector (pEGFP-N1) that contained the *Gm-csf* gene. Then, we determined the effect in mice of intrauterine administration of PBMC compared with transfected PBMC on implantation and the pregnancy rate.

Materials and Methods

All materials used in this study were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise mentioned. Figure 1 provides an overview of the animal experiments.

Animals and ethics

This research was supported by the Research and Ethics Committee of the Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1396.1297). We conducted the experiments with B6D2F1 (C57BL/6 × DBA2) mouse (Royan Institute, Tehran, Iran). Hybrid mice such as B6D2F1 are suitable for reproductive research because of their high fertility rate and the quality and number of released oocytes.

Mice were exposed to controlled lighting and temperature conditions (22–28 °C, 12 h light, and 12 h dark) and received a standard diet of food and water.

Cloning of the *Gm-csf* gene in the pEGFP-N1 expression vector

The *Gm-csf* gene sequence (GenBank accession number NM_009969.4) was incorporated within the pGH cloning vector (Bioneer, Korea) and confirmed by sequencing. Digestion of pGH vector and pEGFP-N1 expression vector was performed by the HindIII restriction enzyme (Takara, Dalian, China). DNA was extracted and purified from the agarose gel using a DNA extraction kit (Qiagen, Hilden, Germany). The $CaCl_2$ transformation method was used to transform the ligated *Gm-csf* and pEGFNP-N1 vector into competent cells. The colonies grew on an agar plate that contained 100 µg/mL kanamycin and were assayed by colony PCR, restriction enzyme digestion and sequencing methods (Sanger sequencing method, ABI3500 genetic analyzer, CA, USA).

Isolation of PBMC from whole blood

Mouse PBMC was isolated as described previously (Emi *et al*., 1991). Next, the PBMC were removed from the interphase layer of the whole blood sample. The cells were washed in PBS. The isolated PBMC (1×10^6 cells/mL) were suspended in Roswell Park Memorial Institute 1640 (RPMI 1640) medium that contained 10% fetal bovine serum (FBS) and then incubated at 37°C and 5% CO_2.

Transfection of pEGFP-N1–*Gm-csf* into PBMC by electroporation

The isolated PBMC (1×10^6 cells/mL) were placed in RPMI 1640 media that contained 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, followed by an overnight incubation. The PBMC cells were subsequently precipitated and resuspended in electroporation buffer (pH 7.2) that contained 90 mM phosphate buffer, 10 mM $MgCl_2$ and 50 mM glucose. Next, 10 µg of the recombinant vector (pEGFP-N1–*Gm-csf* vector: PBMC-pE-GM) per 1×10^6 cells/mL was added to the cell suspension. PBMC were electroporated in 2 mm cuvettes at settings of 500 µs/pulse 2/500 V using an electroporation device (Eppendorf 940000505 Multiporator, NY, USA). The electroporated cells in the plate were coated with fresh medium. Using conditions similar to those mentioned above, we also transfected PBMC with empty vector only (pEGFP-N1 vector: PBMC-pE). The intensity of GFP was observed by fluorescence microscopy (DXM1200F, Nikon; Tokyo, Japan) at 24–72 h post-transfection.

In vitro GM-CSF production by PBMC

PBMC were isolated and transfected with recombinant and empty vector and incubated for 48 h. A cell culture suspension was then prepared, and the concentration of GM-CSF was examined by ELISA (R&D Systems, USA). GM-CSF production in the vector-free PBMC was also assayed.

Endometrium collection

Female BDF1 mice (6–8 weeks old) were mated with vasectomized male BDF1 mice to induce pseudopregnancy. The observation of a vaginal plug was considered to be confirmation of the pseudopregnancy. The pseudopregnant mice were killed by cervical dislocation, and the uterine horns were immediately collected and washed twice with PBS that contained 300 U/mL total of penicillin and streptomycin (Gibco), and the adipose and connective tissue were removed. The uterine horns were cut longitudinally to expose the uterine lumen, and they were placed in collagenase for 1 h in the incubator. Then, the uterine horns were scratched and dissolved in a digestion mix of collagenase–dispase (Sigma), and incubated for 2–3 h. The cell suspension was centrifuged twice at 470*g*, for 5 min at room temperature. Finally, the cells were placed in 25-cm^2 tissue culture flasks in growth media that consisted of DMEM/F12 supplemented with 15% FBS, followed by an incubation for 72 h.

Co-culture of PBMC and endometrium cells

For the co-culture, approximately 1×10^6 endometrium cells were seeded in each single well of a 12-well plate with 2 mL DMEM/F12 medium that contained 100 nmol/L beta-estradiol, 10 nmol/L progesterone and 15% FBS. The plates were placed in an incubator for 72 h, and the cells were washed with media every 2 days. After 72 h, the transfected PBMC and untransfected PBMC (1×10^6 cells) were collected and centrifuged for 5 min at 265*g* at room temperature. The isolated cells were resuspended in DMEM/F12 medium and added to the plate that contained the endometrial cells.

RNA extraction and cDNA synthesis

RNA was extracted from the co-cultured endometrium cells/PBMC using a commercial mini kit (GeneAll Hybrid-R RNA Purification Kit, Seoul, South Korea) according to the manufacturer's instructions. The reverse transcription reaction for each sample was prepared by adding 3 µL random hexamer, 5 µL nuclease free water and 4 µL RNA of each sample to the microtubes at 75°C for 5 min to enable RNA denaturation. The samples were then chilled on ice, followed by the addition of 5× reverse transcription buffer, 200 U reverse transcriptase enzyme, 10 mmol/L dNTP and 10 U RNase inhibitor to the samples. The reverse transcription reaction was performed as follows: 25°C for 10 min, 37°C for 15 min, 42°C for 45 min and 72°C for 10 min. The cDNA was maintained at 4°C for 24 h. PCR of the cDNA was performed in a total volume of 10 µL that contained 5 µL Master Mix (Ampliqon, Stenhuggervej, Odense M Denmark), 1 µL cDNA, 3 µL nuclease-free water and 1 µL primer. Table 1 lists the primers used in this experiment. PCR was performed for the internal control (beta-2 microglobulin [*Beta-2 m*]) and the genes encoding interleukin 6 (*Il6*), leukemia inhibitory factor (*Lif*), vascular endothelial growth factor (*Vegf*), matrix metalloproteinase 9 (*Mmp9*) and *Gm-csf r*, all as follows: 94°C for 5 min, 94°C for 30 s, 60°C for 30 s and 72°C for 45 s for 40 cycles. A final elongation step was carried out at 72°C for 10 min. Each sample was run in duplicate and each experiment was repeated three times. In order to confirm amplification, the samples were run on a 2% agarose gel and visualized by UV light (Hosseini *et al*., 2017).

Table I List of primers employed in quantitative real-time PCR.

Primer name	Sequence 5′ to 3′	Accession no.
Lif	F:CCCATCACCCCTGTAAATGCC	NM_001039537.2
	R:CGCACATAGCTTTTCCACGTTG	
Vegf	F:TCATGCGGATCAAACCTCACC	NM_009506.2
	R:CGGGATTTCTTGCGCTTTCG	
Mmp9	F:CTCTGCATTTCTTCAAGGACGG	NM_013599.4
	R:TACACCCACATTTGACGTCCAG	
Gm-csf r	F:AGTCCTCAACTCCACGGGTCAC	NM_009970.2
	R: GTAGCCACCACGTCGTCCAG	
Il6	F:CTTCCATCCAGTTGCCTTCTTG	NM_001314054.1
	R:AATTAAGCCTCCGACTTGTTGC	
Beta 2m	F: GCTATCCAGAAAACCCCTC	NM_009735.3
	R: CCCGTTCTTCAGCATTTG	

Lif: leukemia inhibitory factor, Vegf: vascular endothelial growth factor, Mmp9: matrix metalloproteinase 9, Gmcsf-r: granulocyte macrophage colony-stimulating factor receptor, Il6: interleukin 6, Beta 2 m: beta-2-microglobulin.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to evaluate the mRNA expression levels of the *Il6*, *Mmp9*, *Vegf*, *Gm-csf r* and *Lif* genes using a StepOne Real-Time PCR System (Thermo Scientific). qRT-PCR was performed in a final volume of the 13-µL reaction mixture that contained 1 mmol/L of each primer (Table I), 1 mmol/L synthesized cDNA, 7 mmol/L DNA Master SYBR Green I Mix (Roche Applied Sciences, Mannheim, Germany) and 4 mmol/L nuclease-free water. The real-time PCR program was run as follows: 95°C for 2 min, denaturation at 95°C for 5 s, 60°C for 30 s, amplification at 72°C for 10 s and 40 cycles of extension. Data normalization was performed using beta-2 m as the internal control gene (Hosseini *et al*., 2017).

IVF procedure

Oocyte collection

Three days before the planned sperm collection and IVF date, 8-week-old female mice were superovulated with 10 IU PMSG, and, after 50 h, they were injected with 10 IU hCG. At 14 h after the hCG injection, cumulus–oocyte complexes (COCs) were separated from the ampulla of the oviduct.

Sperm collection

The male BDF1 mice (8–10-week-old) were euthanized by cervical dislocation. Spermatozoa were isolated from the cauda epididymes and vasa deferentia. The sperm suspension was collected and placed in human tubal fluid medium (HTF) that contained 4 mg/mL bovine serum albumin (BSA) and incubated (37°C, 5% CO_2) for 45 min.

IVF

COCs were inseminated with approximately 1×10^6 sperm/mL in drops of 100 µL of HTF medium and then incubated for 6 h. After the incubation, oocytes were placed in a potassium simplex optimized medium (KSOM) that contained amino acids and 4% BSA. We evaluated the rates of blastocyst formation and embryo development at 24 and 96 h after IVF, respectively (Vahdat-Lasemi *et al*., 2019).

Mouse embryo attachment *in vitro*

The co-cultured PBMC and endometrial cells were seeded in DMEM/F12 medium that contained 100 nmol/L beta-estradiol, 10 nmol/L progesterone and 10% FBS for 2–3 days. Zona pellucida removal was achieved with obtained by acid Tyrode's solution, and zona-free blastocysts were added to the PBMC and endometrial cell co-culture for 72 h and placed in an incubator at 37°C. Blastocyst attachment was checked every 24 h; those that stayed at the same location were defined as attached blastocysts, whereas unattached blastocysts were floating in the medium.

In vivo investigation of embryo implantation and pregnancy

Animal experiment

We established a model of mice embryo implantation dysfunction (EID) using low-dose mifepristone (0.08 mg/0.1 mL) to evaluate the effect of PBMC and GM-CSF on embryo implantation. Female B6D2F1 mice were assigned to five groups: control (without PBMC and mifepristone), EID (without PBMC), EID with PBMC, EID with PBMC-pE and EID with PBMC-pE-GM. The female mice were superovulated with PMSG and hCG (as mentioned in IVF procedure) and then mated with the male mice (ratio 2:1). The presence of a vaginal plug was considered an indication of mating, and that day was determined to be Day 0.5 post coitum. On Day 3.5 post coitum (at 9:00 a.m.), 0.1 mL mifepristone (dissolved in propanediol, 0.08 mg/0.1 mL) was injected s.c. into the four experimental groups described above (Yu *et al*., 2014). The control group did not receive any mifepristone.

Intrauterine transmission of mouse PBMC

Isolated PBMC were transfected with the PBMC-pE-GM and PBMC-pE and incubated for 48 h. Then, 5 µL cells from both of these groups (1×10^6 cells/mL) were surgically transferred to the bilateral cranial part of the uterine horn for the EID with transfected PBMC groups on Day 1.5 post coitum. The EID with PBMC group also received 5 µL of PBMC (1×10^6 cells/mL) by the same procedure on Day 1.5 post coitum. (Fig. 1) (Yu *et al*., 2014).

Tissue collection

Female mice were euthanized by cervical dislocation on day 7.5 post coitum. Both uterine horns were isolated and assessed for the numbers of implantation sites, and the pregnancy rate was determined through the number of pregnant mice in each group.

Statistical analyses

Statistical analyses were performed SPSS version 16 software (IBM, Chicago, IL, USA). Blastocyst attachment *in vitro* and implantation sites *in vivo* was analyzed by one-way ANOVA. The target gene expression rate was analyzed with REST 2009 Software (Qiagen, Hilden, Germany). Data are presented as mean ± SD. $P < 0.05$ indicates statistical significance.

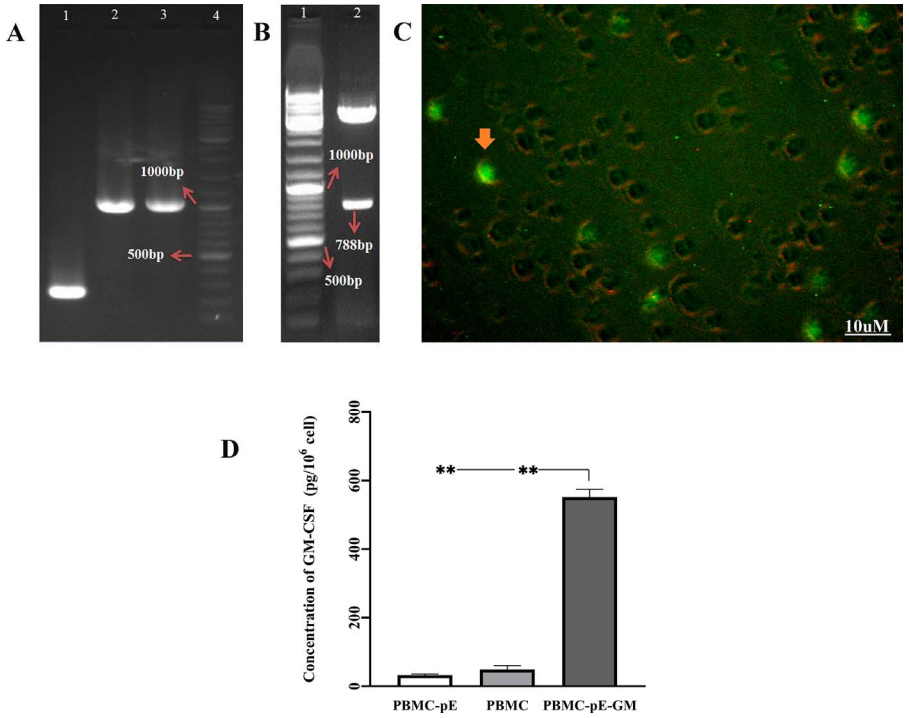

Figure 2 Confirmation of *Gm-csf* gene cloning into the pEGFP-N1 vector. (**A**) Colony PCR with universal PE primers: Lane 1 (negative clone), Lanes 2 and 3 (positive clones), and Lane 4 is the 1-kb DNA ladder. See Supplementary Figure S1 . (**B**) Lane 1: DNA ladder (1 kb). Lane 2: Digestion of the pEGP-N1 vector by HindIII; lower band (788 bp) is *Gm-csf* gene. See Supplementary Figure S2 . (**C**) Fluorescence microscopy image of the mouse PBMC transfected with pEGFP-N1 vector containing the *Gm-csf* gene; arrow indicates transfected PBMC. Scale bar is 10 μm. (**D**) Concentrations of GM-CSF in the different groups (measured by ELISA). Data are mean ± SD. (**$P < 0.01$; ANOVA, Tukey test). PBMC-pE: transfected PBMC with empty pEGFP-N1 vector; PBMC-pE-GM: transfected PBMC with the pEGFP-N1-*Gm-csf* vector.

Results

Cloning of recombinant *Gm-csf*

The synthetic *Gm-csf* gene was successfully cloned into a pGH vector and then inserted into the pEGFP-N1 expression vector. Cloning was confirmed by colony PCR, restriction enzyme digestion with HindIII and sequencing (data not shown). Colony PCR was performed with universal PE primers (F: 5′CGCAAATGGGCGGTAGGCGTG 3′ and R: 5′ GGCCCGTTTACGTCGCCGTCC 3′) for pEGFP-N1. A PCR product of approximately 1000 bp confirmed the successful cloning of the *Gm-csf* gene in the pEGFP-N1 vector (Fig. 2A and Supplementary Fig. S1). The target insert band (788 bp) was shown in the enzyme digestion (Fig. 2B and Supplementary Fig. S2).

Transfection of PBMC and *in vitro* GM-CSF production by PBMC

Visualization of GFP with a contrast microscope showed that the efficiency of the transfection in PBMC-pE-GM was higher than PBMC at 48 h post-transfection. Figure 2C shows transfected PBMC. The cytokine assay indicated that the level of GM-CSF produced in the PBMC-pE-GM group (552 pg/10^6 cells) was significantly higher compared with the PBMC group (57 pg/10^6 cells) and PBMC-pE (34 pg/10^6 cells) ($P < 0.05$) (Fig. 2D).

GM-CSF induced mRNA expression in PBMC co-cultured with endometrial cells

Vegf (5.9-fold), *Mmp9* (11.1-fold), *Lif* (11.4-fold), *Gm-csf r* (11.8-fold) and *Il6* (8.7-fold) mRNA expression levels ($P < 0.05$) in the PBMC-pE-GM group were increased relative to the control group. There was a significant increase in the expression levels of *Vegf* (1.7-fold), *Mmp9* (1.4-fold), *Lif* (1.5-fold), *Gm-csf r* (1.6-fold) and *IL6* (1.2-fold) in PBMC-pE-GM compared to the PBMC ($P < 0.05$). Figure 3 summarizes the mRNA expression levels found in this study.

Endometrium collection and attachment of mice blastocysts to the endometrium-PBMC co-culture

Microscopy images showed that the epithelial cells were cuboidal to columnar-shaped, and the stromal cells were spindle-shaped (Fig. 4A). Table II demonstrates the developmental competence rate for embryos in the IVF experiment. In total, 123/150 (82%) embryos reached blastocyst stage. Attachment and migration of the blastocysts occurred within 24–72 h (Fig. 4B–D). The percentages of blastocysts that attached were 77.8 ± 6.9% (PBMC-pE-GM), 51 ± 3.6% (PBMC), 34.5 ± 2.3 (PBMC-pE) and 31.1 ± 1.92% (control) (Fig. 4E). Our data showed that the rate of attachment in the PBMC-pE-GM group was

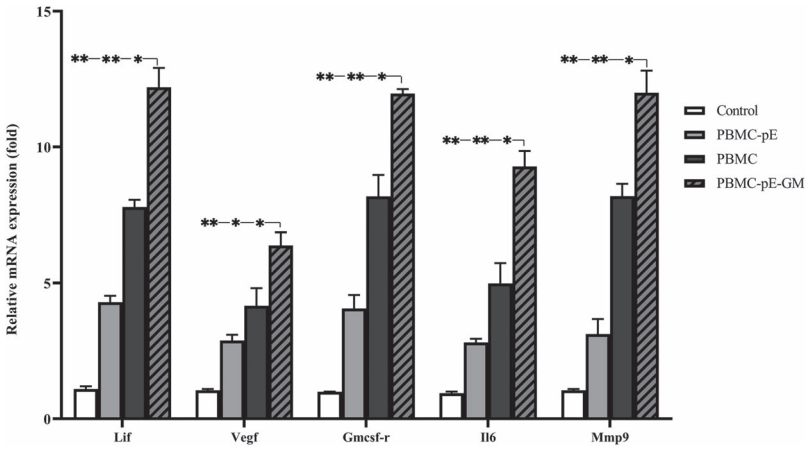

Figure 3 The effects of GM-CSF and PBMC on mRNA expression levels in the mouse endometrium *in vitro*. Expression level genes detected using quantitative real-time PCR. Data are mean ± SD. (*$P < 0.05$; **$P < 0.01$; ANOVA, Tukey test). Lif: leukemia inhibitory factor, Vegf: vascular endothelial growth factor, Mmp9: matrix metalloproteinase 9, Gm-csf r: granulocyte macrophage colony-stimulating factor receptor, Il6: interleukin 6, Beta 2m: beta-2-microglobulin.

higher than the other groups (control: $P < 0.01$, and PBMC-pE and PBMC groups: $P < 0.05$).

Effect of GM-CSF and PBMC on implantation sites and pregnancy rate *in vivo*

The rate of pregnancy in the EID with the PBMC-pE-GM group (89%) was higher compared with the EID with PBMC (75%), EID with PBMC-pE (58%), control (79%) and EID (16%) groups ($P < 0.01$). After intrauterine transmission of PBMC that contained the produced GM-CSF, the number of implantation sites (Fig. 5E) increased compared with the PBMC group ($P < 0.05$), EID group ($P < 0.01$) as well as the control and PBMC-pE groups ($P < 0.05$). Examples of implanted embryos in the different groups are shown in Figure 5A–D.

Discussion

Despite many advances in reproductive techniques, pregnancy rates remain low in infertile women. One factor that plays a key role in embryo implantation is maternal-fetal immune tolerance (Mor *et al.*, 2011; Yakin *et al.*, 2019). Identification of immune cells and molecules that mediate implantation of the embryo in the uterus could facilitate the development of novel therapies and may improve responses to currently available therapies (Tabibzadeh & Babaknia, 1995; Hashii *et al.*, 1998; Norwitz *et al.*, 2001; Dimitriadis *et al.*, 2005; Chaouat *et al.*, 2007). The data from the current study shows that intrauterine transmission of PBMC and PBMC with *Gm-csf* has a positive impact on blastocyst implantation in the uterus and on pregnancy rates; GM-CSF and PBMC impact the mRNA levels of some cytokines that are known to enhance embryo attachment; and PBMC and the GM-CSF protein may improve endometrial receptivity, at least by increasing the production of VEGF and LIF proteins. PBMC mostly consist of mononuclear cells, including lymphocytes and monocytes, which regulate immune and inflammatory responses through cytokine secretion (Robertson *et al.*, 2000; Yu *et al.*, 2014; Yu *et al.*, 2015). Previous studies have shown that PBMC induce mRNA expressions of several inflammatory cytokines, including *IL1*, *IL6* and tumor necrosis factor alpha, which are important for primary immune responses. These inflammatory cytokines induce endometrial differentiation and boost embryo implantation (Cohen *et al.*, 2006; Yang *et al.*, 2016; Dobaño *et al.*, 2018).

We observed increased mRNA expression levels of *Il6*, *Vegf*, *Mmp9* and the *Gm-csf r* in the mouse uterus after transmission of PBMC and GM-CSF in endometrial cells. The concurrent immune system impairment may be a critical reason for an inappropriate quality of oocytes, defective ovulation and progression in pregnancy or possibly loss of pregnancy (Cohen *et al.*, 2006; Lee *et al.*, 2011). An inadequate level of GM-CSF can impair the excitation of T-cellular tolerance at the beginning of pregnancy and impair implantation in pregnancy. It has been demonstrated that leukocyte employment in the immune system is a result of elevated expression of the mRNA transcript of *GM-CSF R* in the uterus of human and mice. This process stimulates embryo invasion and inflammatory responses in the early days of the pregnancy (Rosendaal, 1975; Sjoblom *et al.*, 1999; Robertson *et al.*, 2000; Okitsu *et al.*, 2011b; Tevkin *et al.*, 2014). In our study, it was found that transfected PBMC could promote expression of the *Gm-csf r*.

Inflammatory cytokines, as well as GM-CSF, are effective in regulating the *MMP9* and *MMP2* genes. These two genes increase the capability of human embryos to attach in the endometrium (Nakayama *et al.*, 2002; Nissi *et al.*, 2013). Stimulation of trophoblast cell migration and invasion using cytokines can affect the expression level integrins, such as integrin $\beta 1$ (*ITGB1*) and *MMPs* (Robertson *et al.*, 2000; Furmento *et al.*, 2016). It is reported that granulocyte-colony stimulating factor, through an increase in expression of integrin $\beta 1$, stimulated Swan 71 cell (trophoblast cell line) migration, which can help placental development in human (Robertson *et al.*, 2000; Furmento *et al.*, 2016). In our study, it was found that transfected PBMC could promote mRNA expression levels of the *Mmp9* gene. Our finding confirms the data of previous studies. Also, we showed that intrauterine transmission

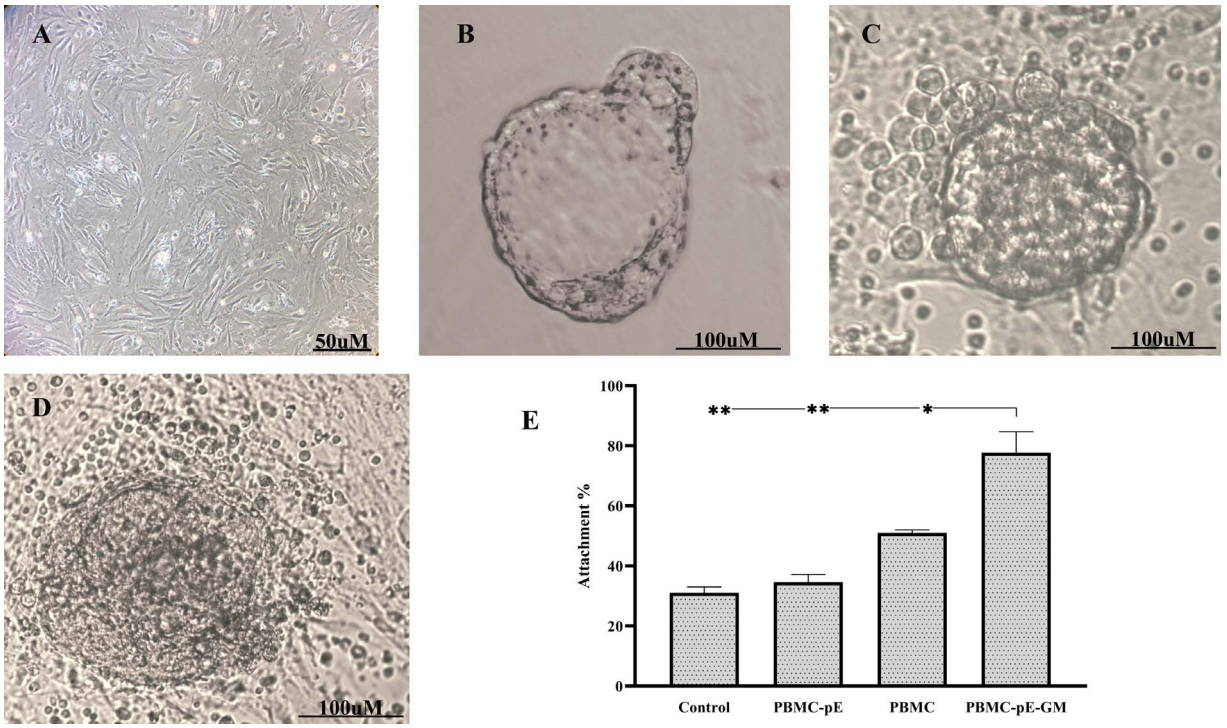

Figure 4 Mouse blastocyst attachment in the *in vitro* co-culture of endometrial cells and PBMC. (**A**) Microscopy images of epithelial and stromal cells from mouse endometrium. Scale bar is 50 μm. (**B**–**D**) Progression of the blastocyst attachment steps (attachment to migration) is shown. Scale bar is 100 μm. (**E**) The percentage of attachment in the different study groups. Data are mean ± SD. (**$P < 0.01$; ANOVA, Tukey test).

Table II Developmental competence rate for mouse embryos after IVF.

Group	No. of oocytes	Two-cell stage	Four-cell stage	Eight-cell stage	Morula	Blastocysts
IVF	150	137 (91.3%)	134 (89.3%)	129 (86%)	125 (83.3%)	123 (82%)

of GM-CSF and PBMC before embryo implantation increased the pregnancy rate. To understand the mechanism of this finding, we examined the rate of implantation *in vitro* and *in vivo* and studied mRNA expression levels of *Vegf* and *Lif*, which are necessary for embryo implantation (Dimitriadis *et al*., 2005; Yu *et al*., 2014). Several studies have determined that LIF is crucial in embryo implantation and could be used as a marker of endometrial receptivity (Rockwell *et al*., 2002; Kondera-Anasz *et al*., 2004; Chaouat *et al*., 2007; Yu *et al*., 2015). VEGF is a vascular permeability factor that affects endometrial proliferation and differentiation at the site of embryo implantation in mice and marmosets (Fujita *et al*., 1998; Norwitz *et al*., 2001; Rowe *et al*., 2003; Salleh & Giribabu, 2014). In rodents, it has been demonstrated that permeability and uterine edema (critical for implantation) are controlled by VEGF. On the other hand, inhibition of VEGF has been shown to stop implantation (Fujita *et al*., 1998; Li *et al*., 2002). The results of the present study showed that mRNA expression of *Vegf* and *Lif* in the mouse endometrium were remarkably up-regulated in both the PBMC and transfected PBMC groups relative to the control. These results indicate that PBMC and GM-CSF may potentially improve uterine receptivity (Rosendaal, 1975; Sjoblom *et al*., 1999; Ideta *et al*., 2010; Yu *et al*., 2015). We observed that the pregnancy rate and embryo implantation in the control group were lower than the other groups in this study. However, both the pregnancy rate and embryo implantation sites were enhanced after transmission of PBMC and transfected PBMC with recombinant vector into the uterus compared with the untreated control group. These results suggest that the PBMC and transfected PBMC could increase implantation and subsequent pregnancy rates. There are possible mechanisms for the increased rate of embryo implantation by GM-CSF and PBMC. First, PBMC might be involved in the production of progesterone (Hashii *et al*., 1998). We have demonstrated that in the control and PBMC groups, mifepristone caused a delay in glandular secretory differentiation and impairment in decidualization (Yu *et al*., 2014) and this suggested mechanism might cause some deficiency in progesterone production. Researchers have reported that transmission of PBMC to the corpus luteum (CL) in an ovary increased the production of progesterone in these cells to stimulate CL function and enhanced the possibility of connecting the embryo and uterus. Progesterone could increase differentiation and uterine

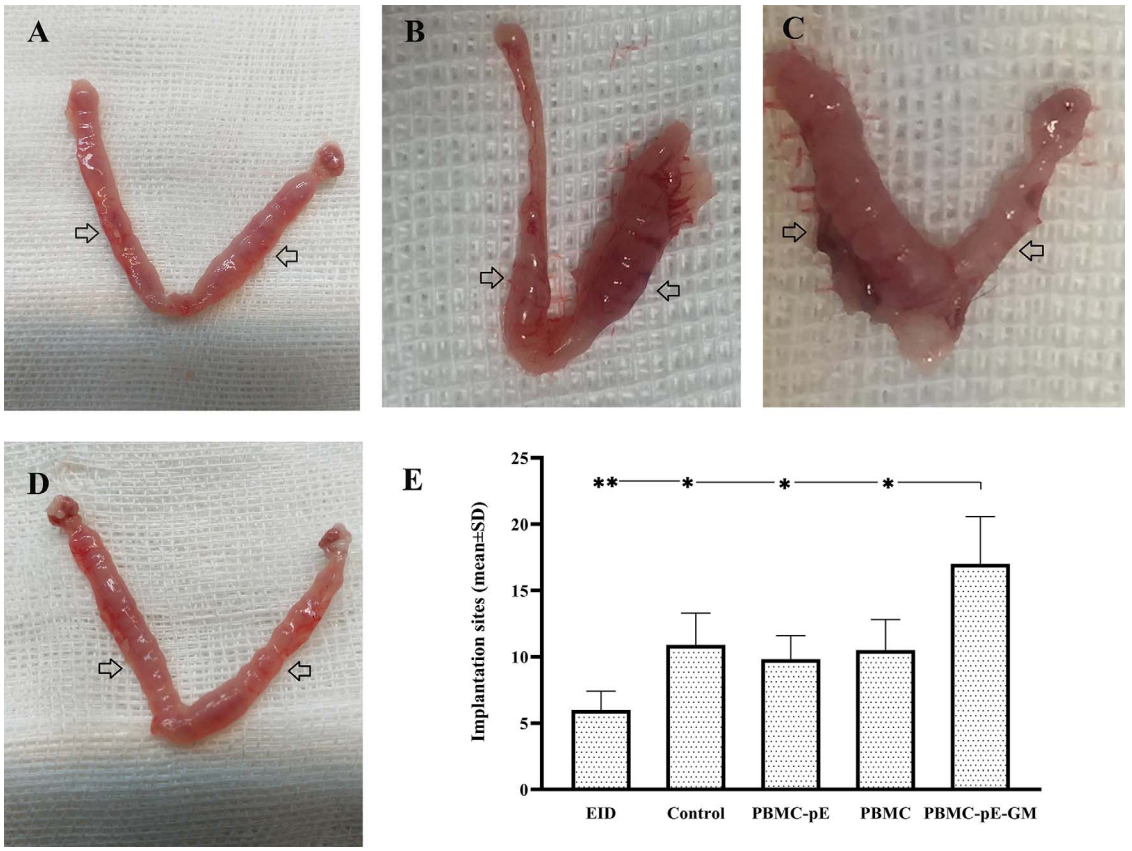

Figure 5 Mouse embryo implantation *in vivo* and number of implantation sites in pregnant mice on post-coitum Day 7.5. (**A**) EID group, 6 implanted embryos; (**B**) EID with PBMC-pE, seven implanted embryos; (**C**) EID with PBMC group, eight implanted embryos; and (**D**) EID with PBMC-pE-GM group, 16 implanted embryos. Arrows indicate implanted embryos. (**E**) The mean number of implantation sites in different groups. (*$P < 0.05$; **$P < 0.01$; ANOVA, Tukey test).

receptivity (Rockwell *et al*., 2002; Yoshioka *et al*., 2006; Yu *et al*., 2015). Second, GM-CSF has a chemotactic activity in the uterus, which has both direct and indirect effects. Direct effects are shown through the movement of macrophages and granulocytes, and indirect effects through the expression of cell adhesion molecules in local endothelial cells (Rosendaal, 1975; Hamilton & Anderson, 2004; Chaouat *et al*., 2007). Finally, transmitted PBMC, by moving from the uterine cavity to the endometrial stromal tissue, may regulate the immunological environment of different regions in the uterus (Yu *et al*., 2014; Yu *et al*., 2015). Studies have demonstrated that with the use of intrauterine administration of PBMC, the early development of pre-attachment bovine conceptus increased, which was attributed to a change in the uterine immunological environment (Fujita *et al*., 1998; Robertson *et al*., 2000; Ideta *et al*., 2010). The contradictory results that have been shown in some studies in human patients (Yu *et al*., 2016) indicate no significant difference in the rate of implantation, clinical pregnancy, and live birth rate between PBMC-treated and control groups. Intrauterine administration of PBMC fails to increase live birth rate when compared to control in women with < 3 implantation failures, which could be a limitation for using this method (Wu *et al*., 2019; Yakin *et al*., 2019). However, this limitation may be linked to an immunological dysfunction of the uterine cavity environment in patients (Yu *et al*., 2016; Wu *et al*., 2019; Yakin *et al*., 2019); therefore, not following the embryos in order to evaluate the live birth rate in mice was a limitation of our study. Altogether, our study showed that endometrium immunotherapy with GM-CSF and PBMC before embryo implantation in mice had an effect on implantation and endometrial receptivity. PBMC and GM-CSF could induce the synthesis and secretion of several cytokines involved in endometrial receptivity. Various immune cells and factors contribute to improved endometrial receptivity, many of which are still unknown. Therefore, more detailed studies are needed to investigate these factors and the mechanism(s) of their action.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Acknowledgements

This research was performed in the Cellular and Molecular Biology Research Center of Shahid Beheshti University of Medical Sciences (Tehran, Iran). We would like thank Sara Hosseini for her help.

Authors' roles

M.S. and D.R.: contributions to design, analysis of the data, drafting and revision of the manuscript for this study. M.B.: contributions to design, interpretation of the data. B.K.: contributions to design. M.S., M.B., B.K. and D.R.: conducted final approval of the final manuscript.

Funding

Shahid Beheshti University of Medical Sciences (research code: 11642).

Conflict of interest

The authors report that they have no conflicts of interest to declare.

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