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ORIGINAL ARTICLE

Parvovirus B19 Infection in Human Bone Marrow Mesenchymal Stem Cells Affects Gene Expression of IL-6 and TNF-α and also Affects Hematopoietic Stem Cells Differentiation

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Abstract Parvovirus B19 (B19V) has been known to induce transient erythroid aplasia, cytopenia and aplastic anemia. This virus persists in bone marrow mesenchymal stem cells (HBMSCs) of some immunocompetent individuals several years after primary infection. In B19V infected erythroid progenitor cells, the virus induces transactivation of Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) gene expression. Due the critical role of HBMSCs in bone marrow niche and inhibitory effect of inflammatory cytokines on hematopoiesis, the aim of this study was to investigate the effect of B19V on IL-6 and TNF-α gene expression intransfected cells. In addition we assessed the clonogenicity potential of cord blood CD34+ stem cells that were co-cultured with infected cells. After 24 h of transfection, quantitative mRNA expression of IL-6 and TNF-α was evaluated and human cord blood CD34+ HSC were cultured with the transfected cells. At the end of 7 days of culture, HSCs colony forming units (CFUs) assay was performed. Our findings demonstrated statistically significant (18.1 and 21.9 fold) increase of TNF-α and IL-6 gene expression respectively and decrease in burst forming unit-erythrocyte (BFU-E) and colony forming unit-erythrocyte (CFU-E) enumeration($p < 0.05$). We concluded that, inducing inflammatory cytokines gene expression in B19V-infected HBMSCs might influence on bone marrow microenvironment and hematopoiesis.

Keywords Colony forming assay · Hematopoietic stem cells · Human bone marrow mesenchymal stem cells · Inflammatory cytokines · Parvovirus B19

Abbreviations

B19V	Parvovirus B19
HBMSCs	Human bone marrow mesenchymal stem cells
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor-α
NS1	Nonstructural protein 1
VP1/VP2	Viral proteins1/2
HHV-6	Human herpes virus 6
VZV	Varicella-zoster virus
EBV	Epstein–Barr virus
CMV	Cytomegalovirus
HAV/HCV	Hepatitis A/C viruses
HIV	Human immunodeficiency virus
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle's medium
CFU-GEMM	Colony forming unit-granulocyte/erythrocyte monocyte/megakaryocyte
CFU-GM	Colony forming unit-granulocyte/monocyte
CFU-G	Colony forming unit-granulocyte
CFU-M	Colony forming unit-monocyte
BFU-E	Burst forming unit-erythrocyte
CFU-E	Colony forming unit-erythrocyte
EPCs	Erythroid progenitor cells

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Introduction

B19V is a common human pathogen of erythrovirus genus and from Parvoviridae family. P antigen is the receptor and KU70/80 and α5β1 integrin are B19V co-receptors. MSCs express these receptors and co-receptors that make them permissive to B19V infection [1, 2]. B19V-DNA encodes NS1 protein with prominent role in replication and transcription, and VP1/2 forming viral capsid [3]. It is not clear why B19V is persisted in many tissues in spite of specific IgG antibodies, B19V DNA CpG methylation and epigenetic regulation are responsive in this persistence [4].

Human bone marrow mesenchymal stem cells (HBMSCs) provides functional and structural support for bone marrow hematopoiesis such as secreting various cytokines and growth factors in bone marrow niche and producing osteoblasts, chondrocytes, adipocytes, endothelial cells and extra cellular matrix. HBMSCs in bone marrow niche support the expansion, quiescence and differentiation of HSCs. Several studies have shown these cells secrete cytokines including IL-6, IL-7, IL-11, IL-12, IL-14, IL-15, macrophage-colony stimulating factor (M-CSF), SCF and FLt3L [5–7].

Viral infection in MSCs considered them as a reservoir and also as the transmission source of these viruses in HBMSCs recipients, so that the safety of applying them in cell therapy should be concerned [8].

Bone marrow defects may occur in HHV-6, VZV, EBV, CMV, HAV, HCV, HIV and dengue infections. Chronic viral infections with these viruses could disrupt blood cells formation and lead to bone marrow failures [9–12].

Recent observations showed the persistence of B19V in HBMSCs of 5% of immunocompetent individuals several years after primary infection. It has been shown chronic B19V infection in immunocompetent people may cause erythroid aplasia, pancytopenia and aplastic anemia [13–20]. HBMSCs infected by B19V-rich plasma showed poor survival, indicating that B19V infection might induce a cytopathic effect on these cells. In addition the infected cells could transmit the virus to hematopoietic cells in vitro [21].

Considering inhibitory effect of inflamatory cytokines on hematopoiesis, the aim of this research was to assess the quantitative changes in IL-6 and TNF-α expression in B19V infected HBMSCs and to evaluate differentiation potential of co-cultured CD34+ HSCs.

Materials and Methods

Cell Culture

HBMSCs were obtained from Pasteur Institute of Iran (Iranian Pasteur Institute's cell bank). Three HBMSC samples from different donors were cultured in DMEM-HG (Gibco, USA), was supplemented with 2 mM of GlutaMAX-ITM (L-alanyl-L-glutamine), 10 U/mL of penicillin,100 mg/mL of streptomycin, and 10% FBS (Gibco, USA). The cultures were incubated at 37 °C and 5% CO_2. The culture medium was changed two times per week for up to 2 weeks. On reaching 80% confluency, cells were trypsinized and replated at a density of about 10^4 cells/cm^2. Cells were expanded for 2–4 passages.

MSCs Immunophenotyping and Transdifferentiation

For HBMSCs characterization about 1×10^5 cells were incubated with appropriate antibodies including monoclonal antibodies against CD90, CD105 (Endoglin or SH2), CD73, CD45 and flowcytometery analysis was performed (FACS CaliburTM, Becton Dickinson, USA). For adipocyte differentiation, 10×10^3 HBMSCs were incubated in 10% FBS-DMEM supplemented with 1 μM of dexamethasone, 200 μM of indomethacin, 1.7 μM of insulin, 500 μM of isobutyl-methyl xanthine. Oil red staining was performed for oil particles in the differentiated cells (Sigma, USA) after 21 days. For osteogenic differentiation, 10×10^3 HBMSCs were cultured in α-MEM supplemented with 10% FBS, 0.1 μM of dexamethasone, 50 μM of ascorbate-phosphates and 10 μM of β-glycerophosphate. After 3 weeks, alizarin red staining was performed for detecting calcium in the differentiated cells (Sigma, USA).

MSCs Nucleofection

Transfection of cells was performed according to Lonza, Amaxa Nucleofector system (Lonza, Switzerland). On reaching 80% confluency, cells were detached by 0.25% trypsin-EDTA (Gibco, USA). 5×10^5 cells were washed with HBSS, per each nucleofection sample. After adding Lonza Nucleofector reagent to the cells and 3μg of pHI0 plasmid nucleofection was performed by using U23 program. After adding 1500 μL of the pre-warmed 10% FBS-DMEM, all contents of suspended cells were divided in 800, 500 and 300 μL in a 48-well plate and were incubated in a humidified incubator with 37 °C/5% CO_2. The same condition was administered for transfecting cells by empty vector as mock (negative control) and Pmax-GFP group. The nucleofection efficacy was assessed after 24 h by flow

cytometry and florescent microscopy for transfected cells with GFP vector.

RNA Extraction and cDNA Synthesis

Total RNA was extracted after 24 h using TriZol Kit (Sigma, USA). Reverse transcription was performed by cDNA Synthesis Kit (Takara, Japan). RT-PCR was performed for NS1 and VP1 genes with specific primers to confirm viral genes expression in infected cells. NS1 (F: GCGGGAACACTACAACAACT, R: GTCCCAGCTTTGT GCAT T AC), VP1 (F: GCAGTC ATGCAAACCTAGA, R: GGCCCAGC TTG TAGCT-CATT) primer sequences were used. Cycle conditions were as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s for a total of 35 cycles (Eppendorf, Germany).

Quantitative Real Time-PCR

In order to compare target genes expression levels in B19V transfected cells and mock cells, qRT-PCR was performed for TNF-α (F: GAGGGTTTGCT ACAACATGGG, R: TCCCCAGGGACCTCTCTCTA) and IL-6 (F: TCAAT-GAGGAGACTTGCCT G, R: GTCAGGGGTGGT-TATTGCAT) by SYBR Green PCR Master Mix. Each reaction was performed using 1 μL total cDNA in 10 μL reaction volume with 0.3 μM of each primer and 5 μL of real-time master mix (Amplicon, Denmark). In each sample GAPDH (F: TTCACCACCACCATGGAGAAGGC, R: GCAGGAGGCATTGCTGATGA), mRNA was used for normalization. After initial temperature at 95 °C for 15 min, amplification and quantification program were as follows: 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 60 s for a total of 40 cycles. Using the Pfaffle method, fold change in the expression of target genes was calculated relative to expression of the GAPDH gene as a housekeeping gene.

$CD34^+$ Cells Isolation and Flowcytometric Analysis

Cord blood (CB) samples were collected from different full-term deliveries immediately after taking parent's written informed consent in each experiment. This Research was approved by the Ethics Committee at Tarbiat Modares University with reference code IR.T-MU.REC0.1394.288. Mononuclear cells were separated from CB by Ficoll-hypac (d = 1.077 g/mL) (Amersham Biosciences, Inc. USA). MiniMACS indirect CD34 Micro Bead Kit (Milteny Biotech, Gladbach, Germany) was employed according to the manufacturer's instructions. The purity of CD34+ cells was evaluated by flow cytometry analysis using an FITC-human CD34 antibody (BD Pharmingen, USA). Non-specific reactions were excluded using isotype controls. The isolated CD34+ cells were collected and were transferred into serum-free stemline II expansion medium (Sigma-Aldrich, USA) supplemented with 10 ng/mL of TPO, 25 ng/mL of stem cell factor (Peprotech, USA), 25 ng/mL of Flt-3L (Stem Cell Technologies, Canada), 2 mmol/l L-of glutamine and 1X antibiotics. The purity of isolated HSCs was analyzed by flow cytometry (FACSCalibur TM, Becton Dickinson, USA).

Co-culture with Transfected Cells

After 24 h of Nucleofection, the three wells of transfected cells were observed by phase contrast microscopy. 20×10^3 of fresh UCB $CD34^+$ HSC in supplemented serum-free medium (stemline II, Sigma-Aldrich) was added to the well with 70% of confluency and was incubated in a humidified incubator with 37 °C/5% CO_2 for 7 days.

Colony Forming Cells Assay

1×10^3 HSCs harvested from the co-cultures after 7 days. The HSCs were cultured on central well plates in a cytokine-supplemented methylcellulose media (MethoCultH4434 classic with cytokine, Stem cell Technologies, Canada) + 2% FBS in Iscove's Modified Dulbecco's Medium (IMDM) following the manufacturer's instructions to enumerate CFCs. After 14 days incubation in 37 °C /5% CO_2CFC assay was performed. CFUs consisting more than 50 cells were counted as colonies. The experiments were performed in duplicate in 3 independent experiments.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism software version 6.0 (Graph Pad Software Inc., San Diego, CA). A paired Student's *t* test was employed to analyze the data. All quantitative measurements were performed at least in three independent experiments.

Results

Characterization of Isolated HBMSCs

Immunophenotyping analysis of HBMSCs showed the cells were positive for CD73, CD90 and CD105 markers and negative for CD45 by flow cytometry analyzer. Oil red and alizarin red staining were positive for differentiated adipocyte and osteocyte lineages respectively (Fig. 1). GFP florescent analysis and florescent microscopy image

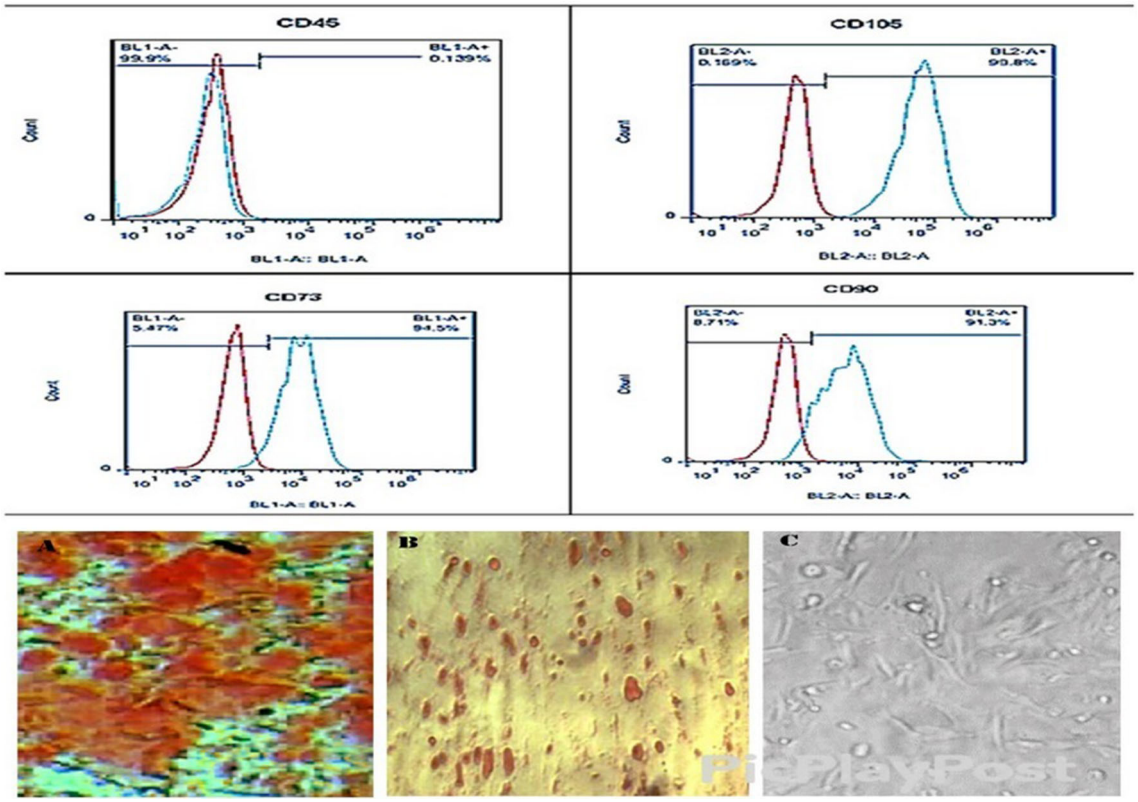

Fig. 1 HBMSCs characterization by flow cytometry analysis. HBMSCs were positive expression for CD90, CD105, CD73 markers and negative for CD45 as hematopoietic marker. Transdifferentiation of HBMSCs into adipocytes and osteoblasts (× 400). The osteoblasts and adipocytes were positive for Alizarin red staining (**a**) and oil red O-staining (**b**) respectively. Undifferentiated HBMSCs show spindle-shape morphology under phase contrast microscopy (**c**)

showed high nucleofection efficiency (Fig. 2). HBMSCs RT-PCR detection for NS1 and VP1 mRNAs showed expression of the viral genes in B19V-transfected cells, NS1 and VP1genes have negative expression in mock (Fig. 3).

CD34+ HSCs Flow Cytometry Analysis

To quantify stem/progenitor cells population CD34 as HSCs marker wasenumerated. CD34+ cell purity was evaluated by flow cytometry analysis using a FITC human CD34 antibody and was > 90% in all cases (Fig. 4).

Colony Formation Assay

The harvested CD34+ cells that were co-cultured with the transfected cells, have the ability to produce clonogenic progenitor cells: CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E and CFU-(E) (Fig. 5) Significant decrease was obtained in BFU-E and CFU-E colony formation for HSCs co-cultured with B19V-transfected cells compared to HSCs co-cultured with mock cells. The mean decrease which was observed in BFU-E and CFU-E of B19V-transfected HBMSCs cultures, compared to mock, was equal to 10.0 ± 1.0 and 6.7 ± 2.1, respectively ($p < 0.05$) (Fig. 6). No significant change was observed in CFU-GEMM, CFU-GM and CFU-G and CFU-M colonies enumeration ($p < 0.05$).

TNF-α and IL-6 Gene Expression

mRNA expression of IL-6 and TNF-α in pHI0-positive HBMSCs were compared to mock by qRT-PCR detecting 24 h after nucleofection. Fold change regarding genes expression of TNF-α and IL-6 was equal to 18.1 and 21.9 ($p < 0.05\%$) respectively (Fig. 7). GAPDH delta CT was used to normalize relative target genes expression quantification.

Discussion

The present study was designed to evaluate IL-6 and TNF-α transcriptional expression as inflammatory cytokines in B19V-transfected HBMSCs. In addition, colony formation assay was performed to analyze clonogenicity potential of

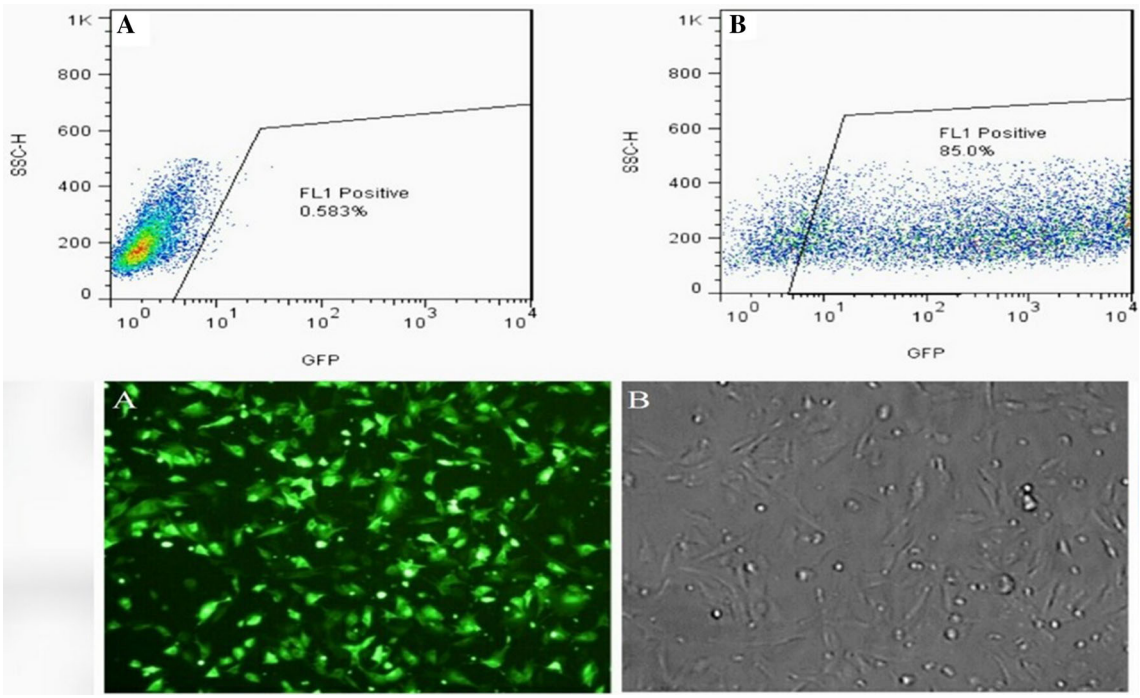

Fig. 2 Transfection efficiency > 85% was obtained for GFP transfected MSCs. Fluorescence microscopy images showed > 80% of fluorescent positive cells that was in parallel to the flowcytometery analysis results

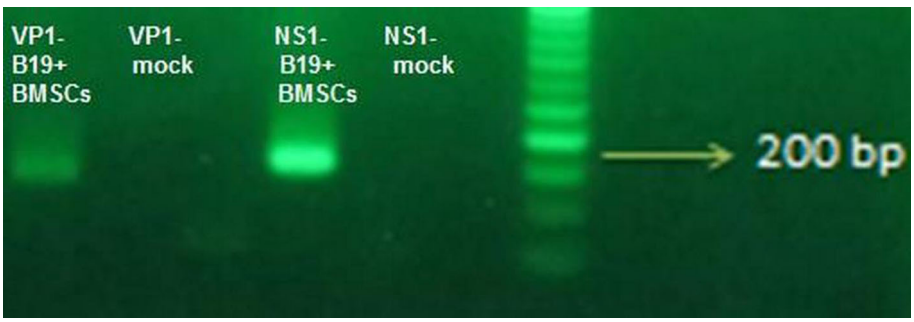

Fig. 3 The gene expression of NS1 and VP1 in HBMSCs before and after transfection. The genes have positive expression in B19V-transfected HBMSCs and negative expression in mock and HBMSCs before transfection

the co-cultured CD34+ HSCs. The results showed significantly increased TNF-α and IL-6 gene expression in B19V-transfected cells ($p < 0.05\%$). Compared to mock, mean fold change in gene expression of TNF-α and IL-6 was equal to 18.1 and 21.9, respectively.

In vitro studies have confirmed the B19V specific tropism to erythroid lineage cells expanded from human bone marrow cultures [11]. Previous studies showed that B19V can inhibit late erythroid progenitor cells (EPCs) and the colonies production of more primitive BFU-E progenitors, but does not influence other hematopoietic progenitors strongly [22].

In our experiments expanded CD34+ in both groups, co-cultured HSCs with B19V transfected cells and mock cells, had the ability to produce clonogenic progenitor cells. Our results showed that co-cultured HSCs with B19V-infected cells reduced the production of erythroid lineage colonies. Statistically significant decrease in BFU-E and CFU-E colonies of HSCs that co-cultured with B19V-transfected cells compared to the HSCs co-cultured with mock cells was observed (10.0 ± 1.0 and 6.7 ± 2.1, respectively) ($^*p < 0.05$). The colony formation analysis of other hematopoietic lineages showed no significant change in their clonogenicity potential ($p < 0.05$).

Several studies support the induction of different cytokines secretion profile in immune and non-immune cells occurring in various viral infections [23]. It is accepted that the secretion of inflammatory cytokines such as IL-1β, TNF-α, interferon (IFN), and IL-6 is common in various viral infections [24, 25]. IL-6 and TNF-α act as inflammatory cytokines and have considerable overlap regarding their effector functions. These cytokines involve in the regulation of immune and inflammatory responses, and influence many bone marrow functions [26]. Studies

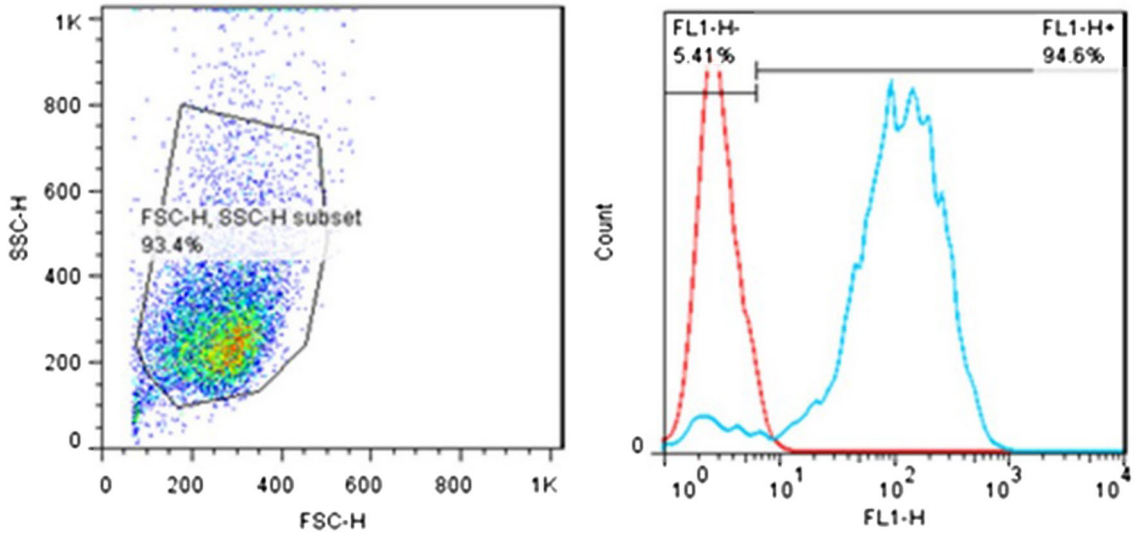

Fig. 4 Flow cytometry analysis of the UCB-HSCs. Flow cytometric analysis showed > 90% of the purified HSCs were positive for CD34 as specific marker of hematopoietic stem cells

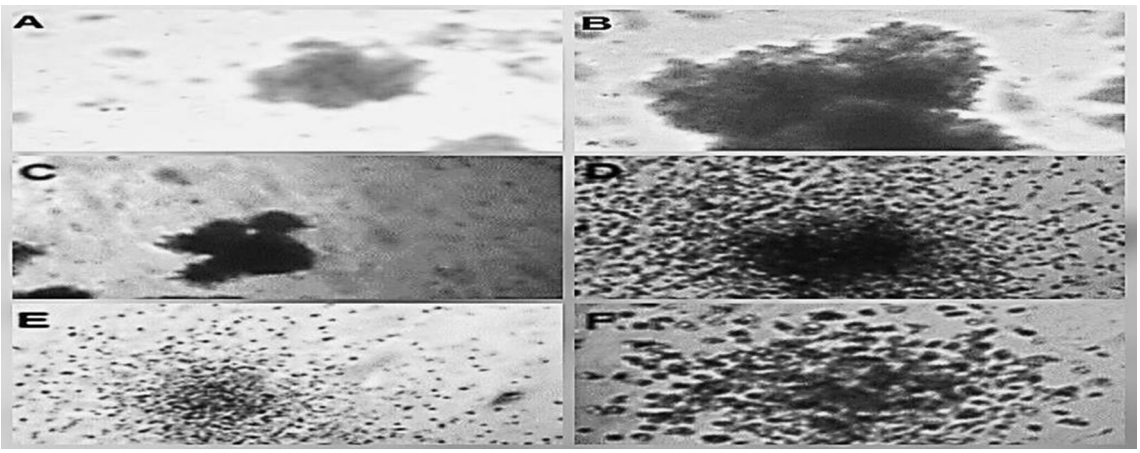

Fig. 5 Morphology of colonies cultured 14 days in MethoCult H4434 medium. **a** Burst forming unit-erythroid (BFU-E) **b** colony forming unit-erythroid (CFU- E) **c** colony forming unit -granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) **d** colony forming unit-granulocyte, monocyte (CFU-GM) **e** colony forming unit-granulocyte (CFU-G) **f** colony forming unit -monocyte (CFU-M) (× 400)

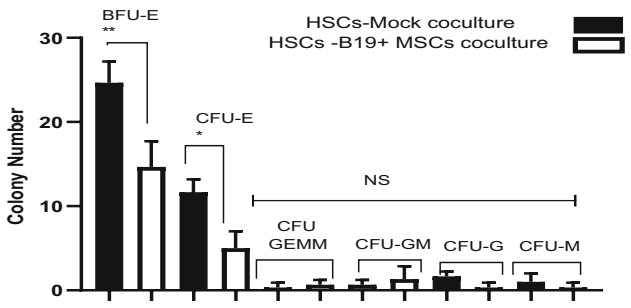

Fig. 6 The mean fold change of mRNA expression in TNF-α, IL-6 compare to mock by real time PCR (N = 3; $p < 0.05$: significant). Data are normalized to GAPGH expression levels

reported that, in some viral infections, altered cytokines secretion or death of bone marrow stromal cells may impair blood cells formation [27]. Given that, HBMSCs play an important role in hematopoiesis by secretion of effective cytokines in bone marrow niche, dysregulation of cytokines expression may influence hematopoiesis [5–7].

Different studies have shown that, B19V–NS1 protein transactivates IL-6 and TNF-α and induces up-regulation of these cytokine expressions in the infected cells [28, 29]. NS1 has been reported to be bound directly to the B19V promoter (p6) and Sp1/Sp3 transcription factors and act as a transcription regulator [30].

Both in vivo and in vitro studies have been shown that, TNF-α is a negative regulator of erythropoiesis and it can

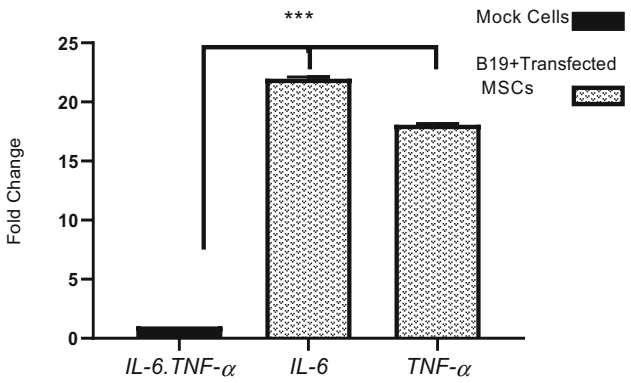

Fig. 7 Colony number change in HSCs cocultured with mock and B19 transfected MSCs. Results of clonogenic assay of cord blood CD34+ after 14 days. The experiments performed in triplicate in three independent experiments. Error bar represent SD. $p < 0.05$: significant

inhibit erythrocyte production [31]. Watanabe et al. [32] showed that TNF-α might play an important role in induction of pancytopenia. The researches about IL-6 effect on hematopoietic progenitor cells have revealed that administration of IL-6 induces anemia, in vivo [33]. Other studies reported that IL-6 treatment has low effect on circulating blood progenitor cells [34]. Another study demonstrated no changes in amount of circulating GM-CFU, BFU-E or CFU-GEMM progenitors after IL-6 administration [35]. Pavese et al. [36] indicated that TNF-α and IL-6 may inhibit bone marrow erythropoiesis in anemic cancer patients and lead to recombinant human erythropoietin resistance. This result can be used in order to explain BM failure in chronic B19V infections. In summary, our study demonstrated the decrease in erythroid colony formation of HSCs cocultured with B19V-transfected cells. These results showed that, B19V could induce inflammatory cytokines in HBMSCs and it may inhibit erythropoiesis.

Conclusion

In summary, our study demonstrated the decrease in erythroid colony formation of HSCs co-cultured with B19V-transfected cells. These results showed that, B19V could induce inflammatory cytokines in HBMSCs and it may inhibit erythropoiesis.

Study Limitation

A limitation of this study is that we evaluated transcriptional expression of the target genes in the transfected MSCs. Evaluation of the cytokines in the level of protein expression could complete and help to the hypothesis that B19V infected HBMSCs may affect on hematopoiesis.

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Authors' Contribution AA and SK designed the experiments. MBF carried out the experiments, analyzed the data and wrote the manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors report no conflicts of interest in this work.

Ethical Approval This study was approved by the local ethics committee of Tarbiat Modares University of Medical Sciences (Approval Number IR.TMU.REC0.1394.288).

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