

Mesenchymal Stem Cells Impact on COVID-19 Patients Immune System, an Ex Vivo Study

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Research

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Abstract

Current clinical management approaches for COVID-19 patients are generally based on supportive treatment, which mainly includes respiratory support and restricted fluid input, which is currently a subject of much debate. Systemic Inflammation caused by SARS-CoV-2 may be related to various extrapulmonary comorbidities such as cytokine-mediated neuroinflammation leading to both non-neuronal and neurological consequences in COVID-19. Mesenchymal stem cells (MSCs) are adult stem cells with multipotent properties suitable for medical applications that have been reported as potential therapies in the setting of lung diseases. The immunosuppressive properties of MSCs provide a strong rationale to explore their potential beneficial effects on immune events in COVID-19. Multiple *in vivo* studies have demonstrated the capability of MSCs to prevent inflammatory responses and reduce lung damage. Recently, the use of MSCs in treating COVID-19 disease has improved long-term pulmonary function, but the specific mechanisms by which MSCs inhibit the severe inflammatory response induced by SARS-CoV-2 have not been elucidated. To the best of our knowledge, this is the first work describing the regulatory effects of MSCs on peripheral blood mononuclear cells (PBMCs) derived from patients with COVID-19 by measuring the pro-inflammatory and anti-inflammatory cytokines expression and secretion. We also examined the effects on the methylation of genes normally suppressed by DNA methylation in PBMCs. Our result showed that MSCs exerted an immune regulatory function on PBMCs in culture, skewing toward a type-2 response. This occurs by a mechanism consistent with a reduction in inflammatory factors (TNF- α , IL-1 β , IL-6, IL-18, and IFN γ) protein and mRNA expression levels. In contrast, the anti-inflammatory cytokines (IL-4 and IL-10) increased following co-culture with MSCs. Consistent with these findings, the DNA methylation status of these immune genes seemed relevant to their expression pattern, except for GATA3, IL-1 β , and IFN γ genes which showed no significant differences in methylation level between PBMCs with and without MSC exposure. Moreover, in co-culture interaction, MSCs modulated the Th1/Th2 cells in PBMCs compared to unstimulated PBMCs. These data demonstrate that MSCs can exert important immunomodulatory functions that affect virus-associated cytokine storms in pulmonary tissue during the severe respiratory stage.

Introduction

In 2019, a novel coronavirus (SARS-CoV-2) emerged in Wuhan, China, and caused a rapid outbreak throughout the world. Coronavirus disease 2019 (COVID-19) is a new respiratory and systemic illness associated with significant mortality and morbidity that may progress to severe hypoxemia [1]. There are multiple important parameters in the management of the COVID-19 that need optimization. On the clinical front, the key to managing the COVID-19 crisis is to control the spread of the virus and reduce complications in severely ill patients admitted to the intensive care unit (ICU) [2]. Intensive clinical research is ongoing on how to best manage critically ill patients with COVID-19 infection; however, developing therapeutic strategies for the treatment of COVID infection is still an important goal [3]. There are now limited therapies available that include non-specific anti-viral agents to decrease viral load, antibiotics to prevent secondary bacterial infections, and corticosteroids to suppress high-risk

inflammatory reactions, all of which cannot sufficiently ameliorate the severe disease where a robust proinflammatory cytokine signature is present [4]. At present, neither a satisfying therapy nor a preventive cure is available for human COVID-19 infection [5]. The outbreak is ongoing and has posed a serious clinical management challenge that impacts the development of chronic respiratory, cardiac [6], and neurological conditions [7]. It has been appreciated that COVID-19 infections in patients with persistent symptoms (beyond three-month duration) are associated with the chronic inflammatory condition [8]. This suggests that infectious SARS-CoV-2 may be an immune trigger that causes excessive immune responses as seen in autoimmune diseases, thus offering a potential clinical option for therapeutic interventions [8]. The onset of SARS-CoV-2 infection may coincide with changes in innate and adaptive peripheral immune cells that regulate the rate of disease progression in COVID-19 patients with the mild-to-moderate illness. Gene expression profiling of circulating leukocytes has been used to characterize human immune responses to SARS-CoV-2 [9]. It is established that SARS-CoV-2 can evoke a massive systemic inflammatory reaction caused by excessive proinflammatory hypercytokinemia, also known as “cytokine storm” [10]. Virally-induced cytokine release of tumor necrosis factor (TNF), interleukin (IL)-6, IL-1, IL-2, granulocyte-colony stimulating factor (GCSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), interferon- γ inducible protein 10 (IP-10), and monocyte chemoattractant protein 1 (MCP-1), if not treated early, can lead to adverse outcomes such as pulmonary edema, acute respiratory distress syndrome (ARDS), interrupted air exchange, and cardiac injury leading to death [11, 12]. As a result, research on treatment modalities and management of COVID-19 mostly focuses on immunomodulatory approaches to treat symptoms and prevent systemic damage[13]. This has led to urgency in basic science research to recognize the application of recent scientific discoveries to the treatment of patients in the clinical setting. The information gained from such studies will provide a rational approach in managing the challenging situation of the COVID-19. It has been proposed that immunomodulatory and reparative properties of mesenchymal stem cells (MSCs) can be leveraged to mitigate the pulmonary and systemic inflammation characteristic of the COVID-19 disease. MSCs are progenitor cells that appear promising as a novel treatment option for infectious diseases in recent years [14]. MSC-based treatment, the potent candidate in cell therapies, may act to prevent detrimental immune responses to the SARS-CoV-2 infection [15]. There is also interest in the potential of MSC-based therapies to improve the causal factors of ARDS by targeting all pathophysiological components of the disease [15]. From a technical perspective, successful isolation of MSCs has been demonstrated from various tissues like bone marrow, blood, adipose tissue, amniotic fluid, umbilical cord, synovial membrane, and dental pulp [16, 17]. Due to their easy availability, low immunogenicity, and high expansion capacity, applying MSCs have increased attention in cell-based therapy for treating immune-based disorders [4]. A number of clinical trial studies using MSCs have been completed or are ongoing in the treatment of the COVID-19 disease with unmet medical need [18–21]. Results from clinical trials and their therapeutic effects have been published indicating that MSCs provide beneficial effects to regain patients' lung function after COVID-19 infection and to restore the disturbed cytokine balance [22]. At least, it can be considered a complementary therapy to alleviate many of the distressing impacts of COVID-19. However, the underlying ways in which MSCs provide relief to patients with COVID-19 has not been elucidated. Considering that immune cells are associated with the pathology and outcomes of SARS-CoV-2 infected patients and that the MSCs may

have a role in modulating immune-inflammatory function, it is important to validate the immunosuppressive/immunomodulatory properties of MSCs in vitro on PBMCs of COVID-19 patients. Many studies have reported that T cell proliferation and activation from PBMCs are modulated in vitro by co-culture with MSCs [23]. Thus, we co-cultured PBMCs obtained from COVID patients with MSCs to elucidate the effect of MSCs on the inflammatory cytokine production profile and investigate the mediating roles of DNA methylation in this process. In this study, we examined the changes of methylation of DNA cytosines that occur after treatment of PBMCs with exosomes generated from MSCs in order to figure out whether this therapy impacts DNA methylation by measuring the methylation level using the methylation specific polymerase chain reaction (MSP) method and explore the mRNA expression of genes involved in inflammatory pathways including IL-1 β , IL-6, IL-18, IL-4, and IFN- γ , as well as determine how inflammatory cells especially T cells in the PBMCs of COVID-19 patients respond to the co-culture with MSCs in vitro.

Materials And Methods

Patients

This study was approved by the local ethics committee (No. IR.TBZMED.REC.1400.047) of the Tabriz University of Medical Sciences, Iran. A total of 20 patients (female = 10, male = 10) with COVID-19 pneumonia, mean age 45.4 ± 4.8 years old, were recruited, and the peripheral blood sample was obtained from each patient after written informed consent. The COVID-19 diagnosis was confirmed based on laboratory findings, a real-time reverse-transcriptase-polymerase-chain-reaction assay of nasal or pharyngeal swab specimens for SARS-CoV-2, and ultrasound imaging. All participants were free from any current anti-inflammatory pharmaceuticals or immunosuppressants, known HIV infection, and had not taken vitamin supplements one month before the study. The information about subject and evaluated clinical and laboratory parameters are summarized in Table 1.

Table 1
Subjects clinical and laboratory information

Patients (n = 20) clinical information	Patients (n = 20) clinical information		
Age, Years	19–69 (53.3 ± 8.4)		
	White blood cell count, × 10⁹/L		
	< 4	4 (20%)	
	4–10	10 (50%)	
	> 10	6 (30%)	
Sex			
Men	10 (50%)	Lymphocyte count, × 10⁹/L	
		< 1·0	12 (60%)
Women	10 (50%)	≥ 1·0	8 (30%)
Current smoking	4 (20%)	Platelet count, × 10⁹/L	
		< 100	9 (45%)
		≥ 100	11 (55%)
Diabetes	1 (5%)	Creatinine, µmol/L	
		≤ 133	18 (90%)
		> 133	2 (10%)
Hypertension	1 (5%)	Lactate dehydrogenase, U/L	
		≤ 245	14 (70%)
		> 245	6 (30%)
Cardiovascular disease	2 (10%)	Bilateral involvement of chest radiographs	20 (100%)
Chronic kidney disease	0	White blood cell count, × 10⁹/L	
		< 4	4 (20%)
		4–10	10 (50%)
		> 10	6 (30%)
Fever		Lymphocyte count, × 10⁹/L	
< 37.3 °C	2 (10%)	< 1·0	12 (60%)
37.3–38.0 °C	8(40%)	≥ 1·0	8 (30%)
38.1–39.0 °C	7 (35%)		
> 39.0 °C	3 (15%)		

Patients (n = 20) clinical information		Patients (n = 20) clinical information	
Cough	11 (55%)	Platelet count, $\times 10^9/L$	
	< 100		9 (45%)
	≥ 100		11 (55%)
Headache	4 (20%)		
Dyspnea	6 (30%)		

Peripheral blood mononuclear cell (PBMC) preparation and cell culture

PBMCs were separated from fresh blood samples collected in EDTA tubes using Ficoll density gradient centrifugation ($d = 1077\text{g/mL}$; lymphosep, Biosera, UK). PBMCs were collected and washed twice with phosphate-buffered saline (PBS) solution (Sigma-Aldrich, Schnelldorf, Germany) and centrifuged for 15 min at 400 g. The mean number of $1.18 \times 10^6 \pm 0.12$ PBMCs were isolated per ml of whole blood. The blood mononuclear cells were then resuspended 1:1 in PBS solution.

Co-culture of PBMCs with MSCs

Before co-culture, commercially available human bone marrow MSCs (Royan, Iran) were seeded into each well of a 6-well flat-bottom culture plate (5×10^4 cells/well) and cultured in 2 ml of RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS (fetal bovine serum, Invitrogen), 2 mM glutamine, 100 U/ml penicillin and streptomycin. After 6 hour cell adhesion at 37°C and 5% carbon dioxide incubator, the PBMC (1×10^6) from COVID-19 patients were loaded onto the plated MSCs. PBMCs and cultured MSCs were co-cultured for 72h in a medium containing RPMI 1640, supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. The ratio of MSCs to PBMC was 1:10 to investigate the MSC-mediated effects. PBMCs cultured without MSC served as control. After the co-culture for 3 days, the suspended PBMCs and cell-free supernatants were collected separately and kept frozen until further analysis. The effects of MSCs were evaluated by fluorescence-activated cell sorting (FACS) for immunophenotype of PBMCs, enzyme-linked immunosorbent assay (ELISA), and real-time polymerase chain reaction (rt-PCR) for cytokine expression.

Cytokine quantification in the supernatants of PBMCs

The concentrations of anti-inflammatory (IL-4) and pro-inflammatory (IL-1 β , IL-6, IL-18, TNF, and IFN- γ) cytokines were measured in media collected from PBMC cultures before as well as after exposure to MSCs by ELISA assay using the kit (MyBio-Source) according to the manufacturer's instructions.

RNA isolation and complementary DNA synthesis

The samples were processed to determine the mRNA levels of Th1/Th2 cytokines and related genes, including T-bet and GATA-3. According to manufacturer method protocol, total cellular RNA was extracted from cultured peripheral blood cells and PBMCs from COVID-19 patients without administration of MSCs using Qiagen's RNeasy Mini Kit (SinaClon), used in RT-PCR. For comparison, RNA extracted from untreated cells was used as a control. RNA was then reverse transcribed to complementary DNA synthesis for the amplification using complementary DNA synthesis kits (Thermo Fisher Scientific).

Real-time quantitative polymerase chain reaction

The expression of 8 genes involved in the inflammatory pathways was profiled. The purity of extracted RNA was estimated by UV spectrophotometer to determine A260/280 and A260/230 ratios. Quantitative analysis of mRNA expression was performed by SYBR green real-time PCR assay, and the LightCycler™ real-time PCR instrument (Roche Molecular Biochemicals) was used to detect the fluorescence. cDNA (5 µl) was added to each well of a 96-well plate, in a total of 10µl reaction mixture that contained 8µl of SYBR Green and 0.5µM of primers. A melt curve was performed at the end of the PCR. The specific primers used were as presented in Table 2. As a housekeeping gene, β-Actin was used as an internal control, and the amount of all mRNA targets in test samples were normalized to the corresponding β-Actin transcript. The PCR reaction was set up in duplicate for each sample. The sequences of primers have been listed in Table 2.

Table 2
Primer sequence

Gene	Primer	Sequence
IL-1	Forward	ACGATGCACCTGTACGATCA
	Reverse	TCTTCAACACCGCAGGACAG
IL-6	Forward	ACTCACCTCTCAGAACGAATTG
	Reverse	CCATCTTGGAAAGGTTCAGGTTG
IL-4	Forward	CCGTAACAGACATCTTGCTGCC
	Reverse	GAGTGTCCCTCTCATGGTGGCT
IL-18	Forward	GATAGCCAGCCTAGAGGTATGG
	Reverse	CCTTGATGTTATCAGGAGGATTCA
TNF- α	Forward	CAGAGGGAAGAGTTCCCCAG
	Reverse	CCTTGGTCTGGTAGGAGACG
IFN- γ	Forward	GAGTGTGGAGACCATCAAGGAAG
	Reverse	TGCTTGCCTGGACATTCAAGTC
T-bet	Forward	CAACAACCCCTTGCCAAAG
	Reverse	TCCCCAAGCAGTTGACAGT
GATA-3	Forward	ACCACAACCACACTCTGGAGGA
	Reverse	TCGGTTCTGGTCTGGATGCCT
β -actin	Forward	AGAGCTACGAGCTGCCTGAC
	Reverse	AGCACTGTGTTGGCGTACAG

(Abbreviation: IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; IL-18: Interleukin-18; TNF- α : tumor necrosis factor-alpha)

Flow cytometry analysis

After being cultured with MSCs in vitro and before the experiments, the percentages of CD4 + IFN γ + Th1 and CD4 + IL-4 + Th2 cells were assessed in PBMCs by flow cytometry. 5×10^6 of PBMCs were washed with PBS followed by a 5-h incubation with phorbol-12-myristate-13-acetate (PMA) (50 ng/mL) plus ionomycin (0.5mM) at 37°C in a 5% CO₂ humidified incubator. The cells were stimulated with monensin as a chemical stimulator and stained with fluorescence-labeled antibodies against the surface and intracellular markers. The mean fluorescence intensity was measured by FACS and analyzed by Flowjo software.

DNA bisulfite treatment and methylation-specific PCR (MSP)

We next profiled DNA methylation of the 8 immune response-related loci in PBMCs of the two groups. Genomic DNA was extracted from the PBMCs by the ??? kit (Company???) as per the manufacturer's instructions. Total genomic DNA isolated from PBMCs prior to and following MSC was modified by bisulfite treatment, and MSP was conducted to explore the methylation status of the candidate genes at the promoter in PBMCs. Basically, bisulfite conversion of DNA leads to the change of un-methylated cytosine residues to uracil residues, whereas the methylated cytosine is unchanged [24]. According to the standard protocol, the first step, sodium bisulfite treatment of the DNA, was carried out using the Zymo EZ-DNA Methylation Kit. As details, sample DNA (10 µl; 1 µg/µl) and distilled water (35 µl) were added into the tubes. NaOH 2 M (5 µl) was added and mixed well and then incubated for 20 min in 37°C. Hydroquinone (C₆H₆O₂, 30 µl; 100 mM) was added, and the solution pipetted until the color of the content changed into light yellow. After that, sodium bisulfite (NaHSO₃, 3 M; 520 µl) was added to it and incubated for 16 h in 50°C water bath. The DNA from the samples was extracted using a DNA extraction kit (Roche, Germany) according to the instruction used for subsequent PCR analysis. The amplification reaction was performed as the following: 1.5 µl of each forward and reverse primer, 4 µl of bisulfite-treated DNA, and 10 µL of PCR master mix with nuclease-free water to make a total volume of 25 µl. To verify amplification validation, the PCR product from each sample was gel electrophoresed and sequenced.

Statistics

Data were analyzed using SPSS 20.0 and GraphPad Prism 5. The repeated measure ANOVA was employed to assess the main effects of time × treatment interaction. Independent t-test analysis compared the changes in the measurements between the groups. Parametric data expressed as mean ± the standard deviation and statistical significance were defined at p < 0.05.

Results

The effect of MSC treatment of PBMCs on the concentration of cytokines released

For the in vitro model of immune response to MSC effect, PBMCs isolated from the whole blood of COVID-19 patients were co-cultured with MSC. Cytokine levels were measured in supernatants after 24 hours of PBMC culture with MSCs (Fig. 1). The protein levels for the pro-inflammatory cytokines IL-1β (pretreatment, 127.8 ± 82.87 pg/ml versus post-treatment, 90.05 ± 55.65 pg/ml), IL-6 (pretreatment, 135.2 ± 68.33 pg/ml versus post-treatment, 105.4 ± 63.37 pg/ml), IL-18 (pretreatment, 2024 ± 1196 pg/ml versus post-treatment, 1530 ± 986.6 pg/ml), TNFα (pretreatment, 2413 ± 795.6 pg/ml versus post-treatment, 2034 ± 657.4 pg/ml), and IFNγ (pretreatment, 2302 ± 1076 pg/ml versus post-treatment, 1458 ± 981.8 pg/ml) showed robust decreases in response to MSC treatment of PBMCs while the expression of regulatory cytokine IL-4 (pretreatment, 148 ± 80.55 pg/ml versus post-treatment, 212.6 ± 109.9 pg/ml), was significantly up-regulated.

The fold-change in inflammatory molecules mRNA expression in PBMCs co-cultured with MSCs

We then examined whether the altered protein expression of inflammatory molecules was related to the level of their mRNA expression in their PBMCs. The expression level of each protein detected in the untreated cells was assigned a value of 1. The data showed robust induction of humoral responses by MSC characterized by an increase in the Th2-like responses and a concomitant reduction in Th1 cell features. The induction of T-bet (0.5015 ± 0.3237 or < 50% of the pre-treatment value) and IFN- γ (0.4905 ± 0.3385) genes was suppressed, but a marked induction of Th2-associated genes including GATA3 (2.209 ± 0.7415), IL-10 (1.752 ± 0.7069), and IL-4 (1.734 ± 0.9167) was observed after MSC exposure, indicating the predominance of anti-inflammatory phenomena (Fig. 2). Meanwhile, the administration of MSC significantly decreased the up-regulated mRNA levels of TNF- α (0.5565 ± 0.4526), IL-6 (0.5415 ± 0.3064), and IL-18 (0.629 ± 0.3295) compared to pre-treatment levels (value of 1).

DNA methylation data comparison between COVID-19 patients' PBMCs before and after the MSC-exposure

In order to define if the altered expression of those transcripts in the PBMC cultures were due to an alteration in epigenetics, DNA methylation analysis was performed in DNA extracted from PBMCs using the MSP as previously described. We observed an increase in the DNA methylation status of multiple gene promoters not only of pro-inflammatory T-bet (pre-treatment, 34.20 ± 12.66 versus post-treatment, 64.70 ± 16.10), IL-6 (pre-treatment, 32.90 ± 10.33 versus post-treatment, 61.05 ± 20.37), IL-18 (pre-treatment, 29.45 ± 11.35 versus post-treatment, 60.45 ± 19.52), and TNFa (pre-treatment, 22.32 ± 9.54 versus post-treatment, 57.80 ± 20.94) but also of the anti-inflammatory IL-4 (pre-treatment, 27.65 ± 10.66 versus post-treatment, 59.30 ± 16.65) following exposure to MSC (Fig. 3). However, the most significant changes occurred in the TNFa gene, blockade of which causes down-regulation of inflammatory responses [25]. GATA3 (pretreatment, 49.56 ± 11.32 versus post-treatment, 55.95 ± 17.27), IFN- γ (pretreatment, 55.36 ± 11.90 versus post-treatment, 63.55 ± 17.21), and IL-1 β (pretreatment, 55.32 ± 9.90 versus post-treatment, 65.55 ± 18.48), gene methylation levels did increase in response to PBMC co-culture with MSC, but not to levels that were comparable to untreated PBMCs. These results show that the key genes involved in inflammatory immune pathways in COVID-19 were noticeably hypermethylated following MSC treatment.

MSC-mediated Th1/Th2 cell immunomodulation in PBMC

Previous studies show that MSCs can change the helper T-cell balance [26]. To assess the ability of MSCs to inhibit Th1 cells and induce regulatory Th2 cells which are likely to have roles in controlling COVID-19 disease progression, PBMCs isolated from COVID-19 patients were co-cultured with MSCs, and the distribution of immune cell subsets in isolated PBMCs was further examined prior to and after the MSC treatment. Flow cytometry analysis showed that the percentage of CD4 + IFNy + Th1 cells decreased while CD4 + IL-4 + Th2 cells exhibited a significant ($p < 0.05$) increase in PBMCs co-cultured with MSCs compared to PBMCs before co-culture with MSC. These results were consistent with the reduced expression of Th1-related cytokines (IL-1 β , IL-6, and IFNy) and transcription factor (T-bet), and up-

regulation of Th2 cytokines such as IL-4, and IL-10 and its transcription factor (GATA3). We then focused on Th1/Th2 balance by measuring the intracellular cytokine concentration in TH cells. After treatment, the ratio was significantly reduced (from $26.55 \pm 6.579\%$ to $19.44 \pm 3.065\%$). This suggests that MSCs could affect the immune response direction by changes in the level of cytokines towards strengthening the anti-inflammatory function.

Discussion

The SARS-CoV-2 causes various clinical symptoms ranging from asymptomatic or mild to severe, often fatal disease. The disease starts as a simple viral infection, but as COVID-19 evolves, immunologic complications may increase accompanied by the development of cytokine storms, eventually leading to severe organ damage [27]. Immune system function in a precise regulation is vital to eliminate the COVID-19 infection and to stop the disease progression to worse outcomes, but a massive and prolonged anti-viral immune response will result in host tissue damage. Controlling undesired immune responses has serious implications for the medical care of patients with COVID-19. Severely ill patients with SARS-CoV-2 pneumonia develop a hyperimmune state with a dramatic increase in several pro-inflammatory cytokines interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF- α) [28]. The excessive inflammatory response during COVID-19 infection that is the major cause of death in these patients is closely related to the Toll-Like Receptor-binding efficacy of SARS-CoV-2, which causes the release of pro-IL-1 β and inflammasome activation, followed by the production of active IL-1 β as a main mediator of fever and lung inflammation [29]. Uncontrolled activation of TLR4 inflammatory signals has been reported to involve in immunopathological features of COVID-19 infection [30]. TLRs-mediated signaling in humans triggers markedly extensive cytokine and chemokine release [31], which is a characteristic of COVID-19 disease. Many COVID-19 patients have an overproduction of several inflammatory cytokines and chemokines, mainly those released after TLR4 activation, such as IL-1 β , -2, -6, -8, -9, TNFa, G-CSF, GM-CSF, and MIP-1 [32]. Mononuclear cells harvested from peripheral blood of COVID-19 patients show an increased expression of TLR-4 and its downstream effectors, including MyD88, CD14, TRAF6, TIRAP, IRAK1, TICAM-1, and NF- κ B [33]. In a Korean study on COVID-19 patients, Lee et al. [34] showed up-regulation of TNF and IL-1 β in all cell types of PBMCs. PBMCs are a critical biological sample used for studying the effects of new compounds on various human immune cells [35].

Since immune mechanisms often play a role, antivirals alone do not control the disease. They target inflammation via anti-inflammatory therapies to prevent severe complications before multiorgan involvement seems to have successful outcomes. Although non-steroidal anti-inflammatory agents have been effective in treating viral respiratory infections caused by influenza and rhinovirus, they are not recommended in the course of COVID-19 infections [36]. According to the different inflammatory pathways involved, researchers have thought of deploying the kinds of biologic agents targeting cytokines through inhibitors or specific cytokine antagonists such as TNF inhibitors, IL-6R monoclonal antibodies, IL-1 antagonists, or Janus kinase inhibitor (JAK) inhibitors [37]. However, such an approach may not be a very effective measure in controlling cytokine storms since different cytokine networks are closely implicated in the COVID-19 disease [38]. In addition, some of these specific biologics, such as JAK

inhibitors, are known to block JAK signaling, thereby preventing the antiviral activity of interferons that cause adverse consequences for the immune system [37]. Hence, the development of natural substitutes to counteract inflammatory pneumonia while being biocompatible, safe, and cost-effective is of great importance. Due to their anti-inflammatory, immunosuppressive and homing abilities, MSCs have been found to ameliorate conditions like respiratory virus-induced pneumonia [39] that are highly correlated with immune activation.

PBMCs that comprise lymphocytes and monocytes are part of both the innate and adaptive immune functions and have a critical influence in surveillance for infectious threats. PBMCs appear to be used as an experimental model system to study response to viral infections. We thus aimed to work with human PBMCs from immune-active COVID-19 patients. There is also evidence that MSCs can bind to activated immune cells resulting in immune cell loss of function, while in the absence of active immune response, MSCs appear to promote the proliferation and survival of lymphocytes [40]. In addition, modulation of immune inflammation mediated by MSCs is performed by secreting cytokines such as transforming growth factor- β (TGF- β), prostaglandin E2 (PGE2), and other anti-inflammatory factors [41]. MSCs have broad curative effects, including tissue repair, alveolar fluid clearance, control of pulmonary endothelial permeability, and immunomodulation [42]. COVID-19 disease is a complex immune dysfunction where the hallmark is a hyperimmune reaction called the cytokine storm that simultaneously occurs alongside an insufficient immune effector function. Excessive and acute cytokine release of IL-6, IL-2, IL-7, G-CSF, IP-10, MCP-1, and TNFa triggered by SARS-CoV-2 virus directly correlates to lung damage, pulmonary edema, dysfunction of air-exchange, respiratory distress, cardiac injury, and multiorgan failure which eventually causes death. Uncontrolled release of inflammatory mediators, giving rise to a cytokine storm and immunopathology are the leading cause of COVID-19-related ARDS [43]. The key role of MSC is to suppress proinflammatory cytokine production and thus attenuates the cytokine storm in these patients [44]. The present study demonstrates that MSC therapy may dampen the cytokine storm by regulating pro-inflammatory mediators IL-6, IL-1 β , IL-18, and IFNy in PBMCs. We used gene expression analysis by real-time RT-PCR to identify the production of cytokines and the methylation analysis to confirm that these genes are affected when MSC treatment was applied. The methylation of specific cytosine residues in DNA is a major epigenetic mechanism vital in multilevel gene expression regulation. Here we describe DNA methylation alterations that may occur in immune responses of the PBMCs co-cultured with MSCs. DNA methylation changes can alter gene expression patterns of innate and adaptive immunity pathways and thus influence their function. For example, evidence has suggested that DNA methylation can direct transcriptional programs of cytokines, IL-2 and IFNy, and the receptor CCR6 in memory T cells [45–47]. DNA methylation has been found to be strongly correlated with the responsivity of PBMCs to TLR ligands [48]. In this report, we show for the five inflammatory cytokines promoters activated in PBMCs that DNA methylation of these regions was significantly different before and after treatment with MSCs in vitro. The data suggest that incubation with MSC ex vivo enhances the methylation status of the T-box transcription factor T-bet gene in PBMCs, but not the Th2 transcription factor, GATA3. The lineage-defining transcription factors including T-bet and GATA3 has been described as master regulators of the fate of CD4 + T cell subtypes [49]. Naïve CD4 + T cells are plastic cells able to differentiate into T helper 1

(Th1) cells that produce IFNy and TNFa or the Th2 cells that secrete IL-4. T-bet is required to promote Th1 gene expression patterns and the Th1 phenotype. T helper plasticity that is the ability of helper T cells to change gene expression programs or even redifferentiate into various effector T-cell subsets can be augmented under the influence of some stimuli. For instance, cytokines such as type I interferons can induce T-bet production and IFNy secretion in Th2 cells that appear terminally differentiated [50]. Similarly, Th17 cells can switch from IL-17 to IFNy production upon culture with IL-12 [51]. Previous studies show that DNA methylation is a key mechanism for establishing the epigenetic state of signature cytokines loci and the control of plasticity in CD4 + helper T cells [52, 53]. Thus, in contrast to the genome, the epigenome can be reprogrammed by environmental factors. In the current experiment, MSCs increased Th2 and decreased Th1 levels in COVID-19 PBMCs accompanied by decreased Th1/Th2 ratio as compared to baseline (before co-culture) and it can be speculated that the shift from Th1 to Th2 is involved in the mechanism of COVID-19 pneumonia treatment by MSCs. The pattern of produced cytokines confirms this into an anti-inflammatory profile, including increased IL-4 and IL-10 production, up-regulated GATA3 transcription, and hypermethylation of Th1 key transcription factors T-bet locus. Although DNA methylation levels remained unchanged, analysis of amounts of the proinflammatory cytokines IL-1 β and IFN- γ released upon challenge revealed that both cytokines were clearly downregulated. Given that migration of PBMCs leads to accumulation of inflammatory cells in tissues, understanding features linked to inflammatory responses of PBMCs is important as a model to study the beneficial changes induced by MSC therapy. Numerous clinical studies have explored the promising efficacies of mesenchymal stem cells in treating COVID-19 patients. Many clinical trials of MSCs including MSC derivatives for the treatment of COVID-19-induced pneumonia have shown positive results in improving outcome without adverse effects [19, 39, 54].

Conclusions

COVID-19 patient PBMCs appear to respond to MSC therapy when grown in co-cultures by modulating the expression of a large number of genes, including those associated with inflammation. The results with PBMCs from COVID-19 patients here ensure treatment efficacy to prevent the risk of the cytokine storm.

Abbreviations

MSCs (Mesenchymal stem cells), PBMCs (peripheral blood mononuclear cells), TNF (tumor necrosis factor), GCSF (granulocyte-colony stimulating factor), IL (interleukin), IP-10 (interferon- γ inducible protein 10), ARDS (acute respiratory distress syndrome), PBS (phosphate-buffered saline), ELISA (enzyme-linked immunosorbent assay), FACS (fluorescence-activated cell sorting), rt-PCR (real-time polymerase chain reaction), JAK (Janus kinase inhibitor), TGF- β (transforming growth factor- β), PGE2 (prostaglandin E2)

Declarations

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Not applicable.

Authors' contributions

Raede Saraei contributed to the conception and design, manuscript writing, collection and assembly of data, and data analysis and interpretation. Kosar Malekpour contributed to the data analysis and interpretation. Ali Hazrati contributed to the collection and assembly of data and final approval of the manuscript. Behnam Hashemi, Jamshid Gholizadeh Navashenaq, Leila Roshangar, Mehdi Yousefi to the conception and designHossein Samadi kafil, Faroogh Marofi contributed manuscript writing. Majid Ahmadi contributed to the conception and design and final approval of the manuscript.

The authors read and approved the final manuscript.

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Availability of data and materials

The data used to support the findings of this study are included within the article.

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

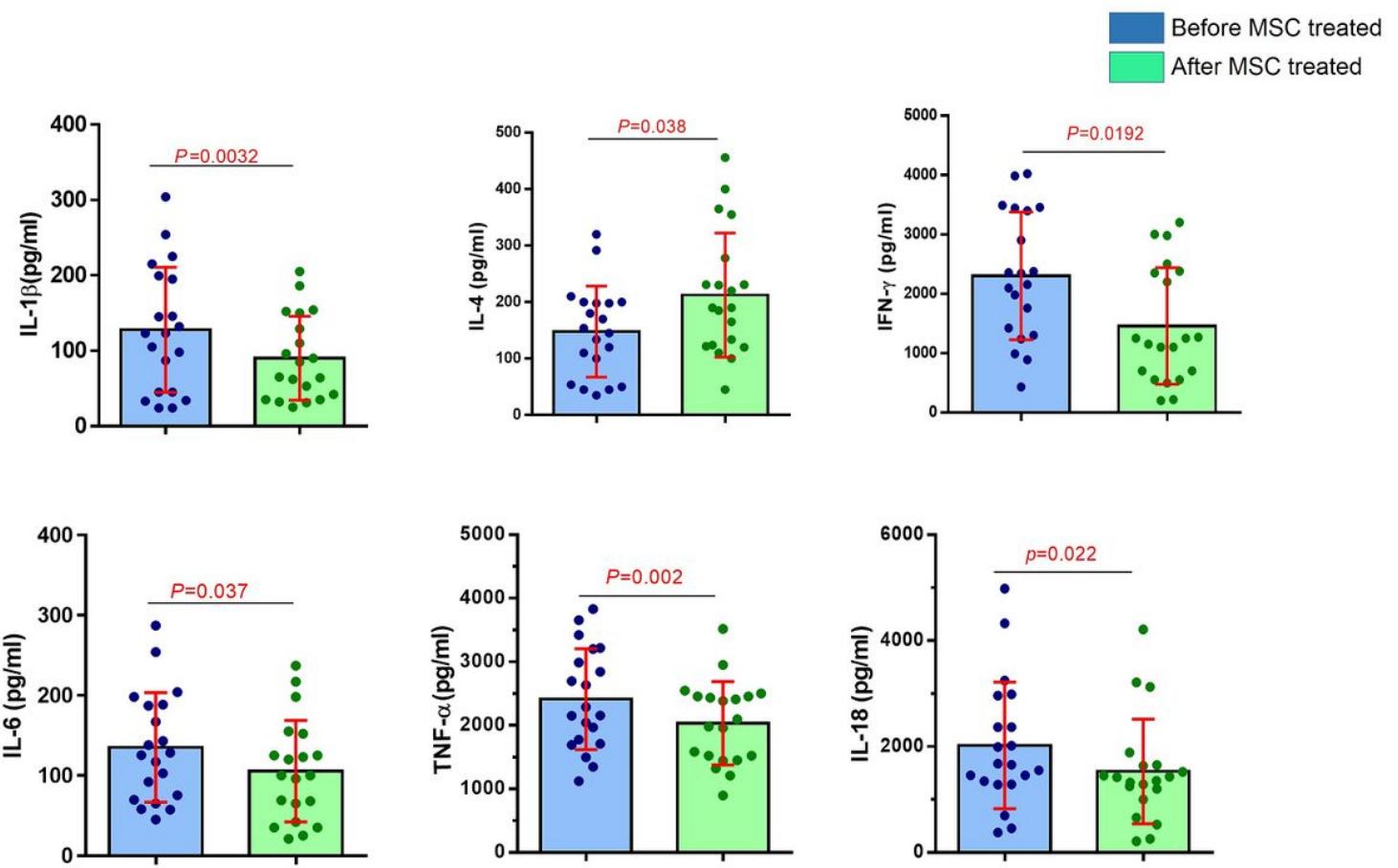


Figure 1

The levels of cytokines secreted in vitro by PBMCs before and after exposure to MSC. Concentrations of pro- and anti-inflammatory cytokines in supernatants of cultures of PBMCs were measured by Elisa. The dots each represent a single specimen examined. IL-1 β , IL-6, IL-18, TNF α , and IFN- γ were significantly decreased following co-culture of PBMCs and MSCs (all P values < 0.05), whereas the levels of IL-4 increased considerably compared to the pre-treatment levels.

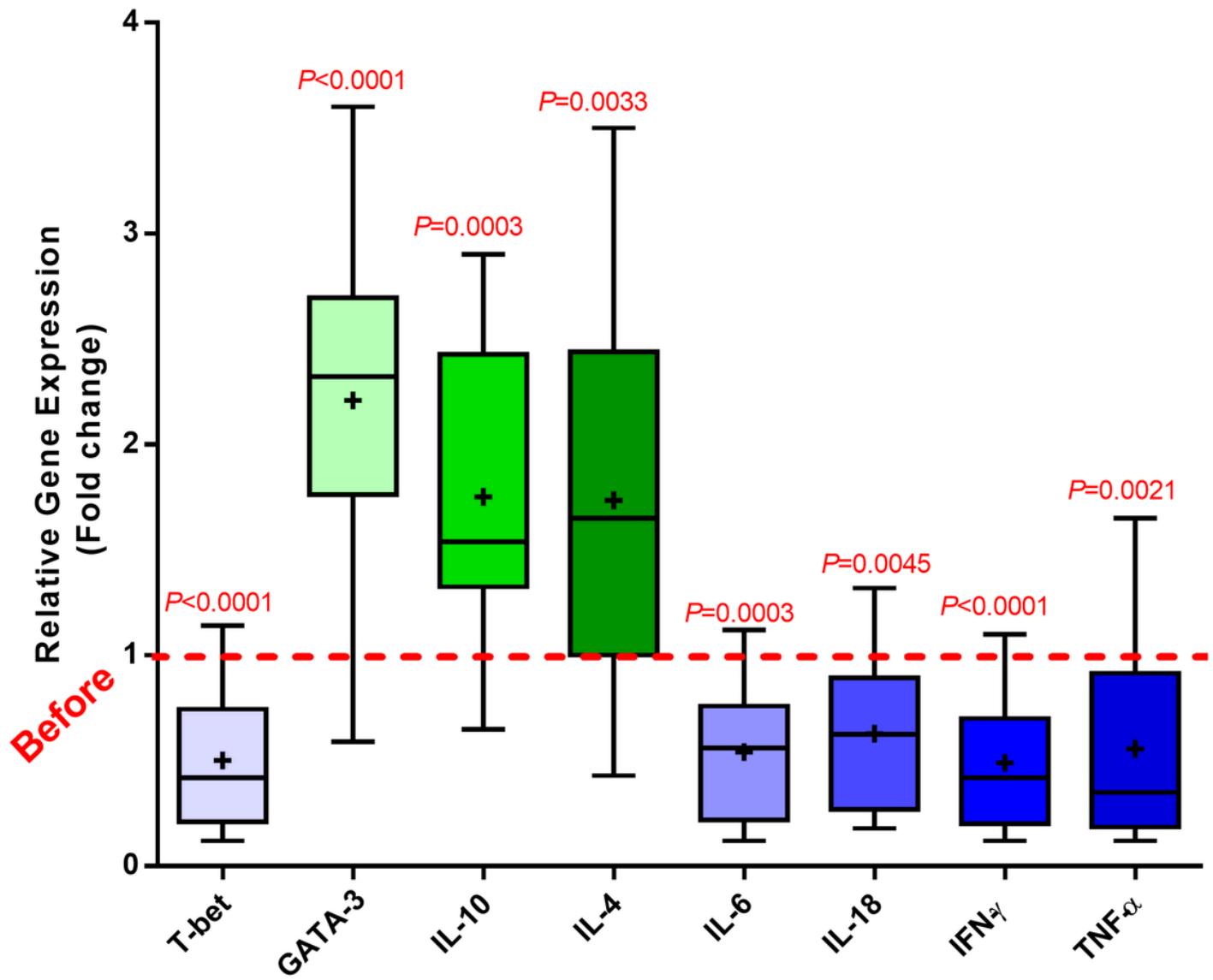


Figure 2

Differences in fold change values of mRNA expression in PBMCs from COVID-19 patients before and after co-culture with MSCs. Th2-specific transcription factor GATA3, and cytokines IL-10, and IL-4 increased in the cultures of COVID-19 PBMCs with MSCs. The maximum fold change was observed for GATA3 gene. A reduced expression of the Th1-related transcripts T-bet, IL-6, IL-18, IFN- γ , and TNF α with a fold change less than 0.5 was observed.

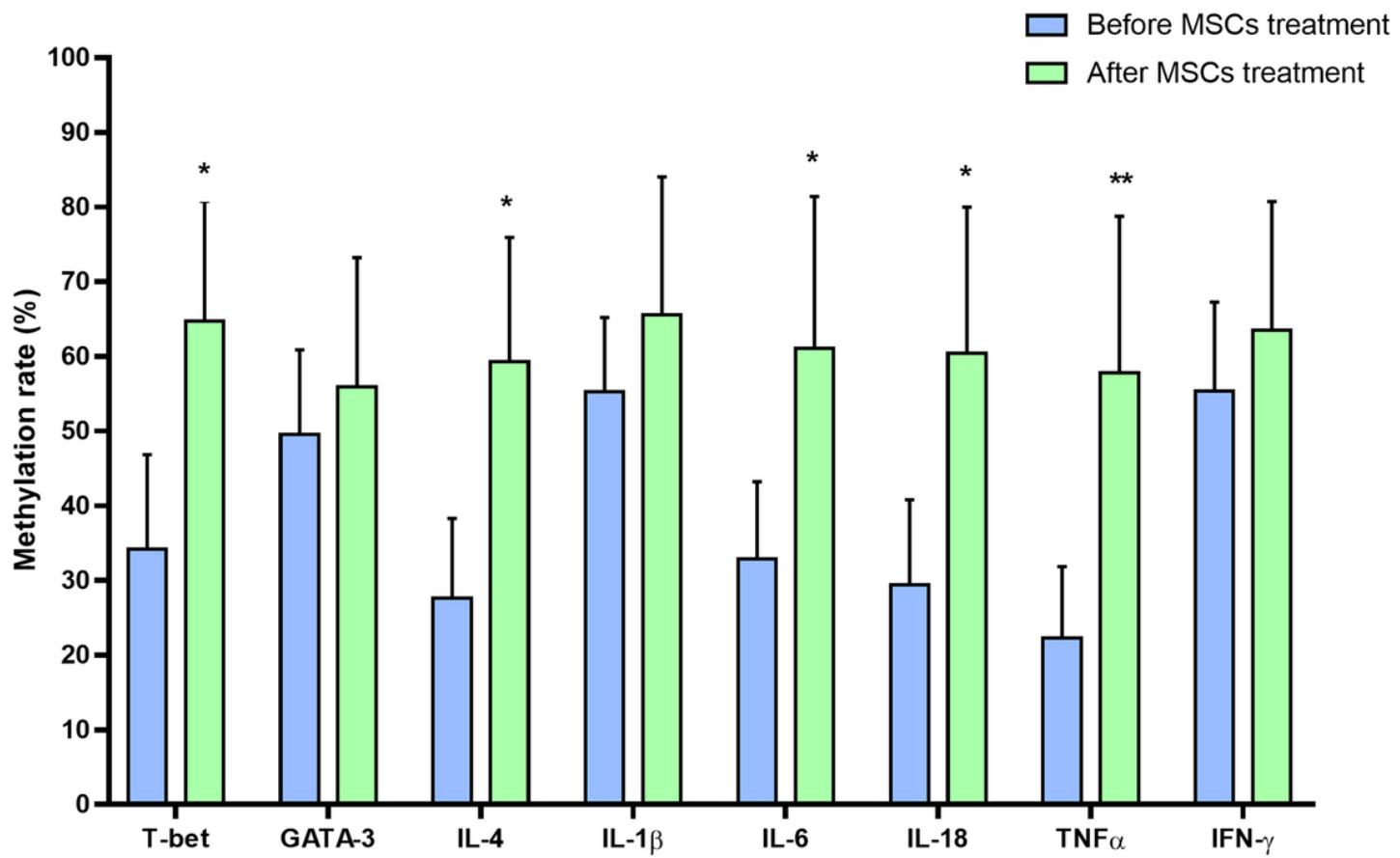


Figure 3

DNA methylation levels of PBMCs from COVID-19 patients before and after co-culture with MSCs. Comparison of methylation rate of the selected proinflammatory genes in the promoter region revealed a higher methylation percentage at IL-6, IL-18, TNF α and T-bet transcription factor compared to baseline (before treatment). PBMCs co-cultured with MSCs had a higher methylation intensity at IFN- γ , IL-1 β , and GATA3 CpG islands rather than that in isolated PBMCs from COVID-19 patients, but this difference was not statistically significant. Notably, the methylation rate of IL-4 gene increased dramatically following PBMC exposure to MSC.

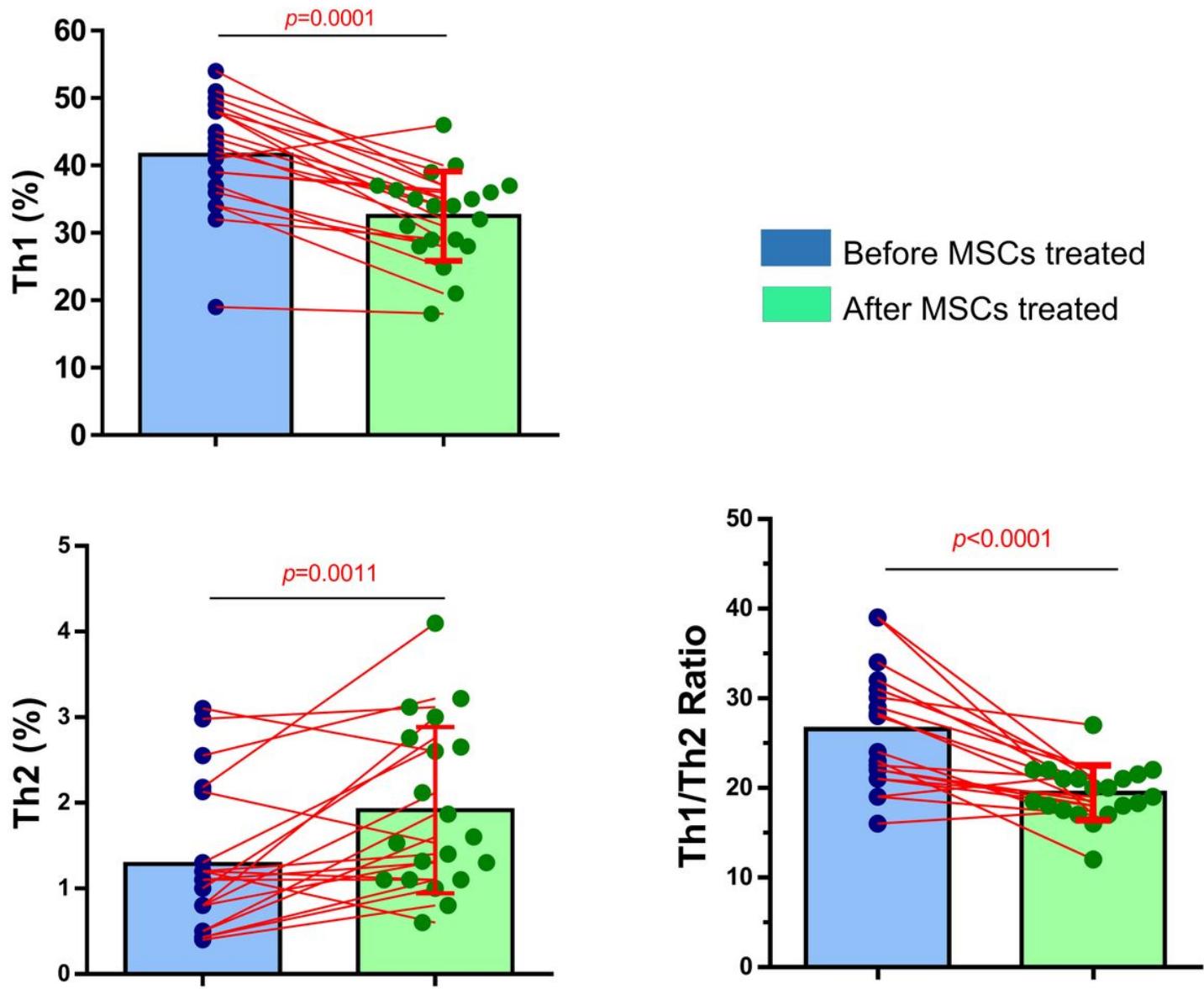


Figure 4

Anti-inflammatory effects of MSCs in vitro through regulation of Th1/Th2 balance. Flow cytometric detection of Th1 (IFN- γ) and Th2 (IL-4) cytokines in PBMCs showed a marked reduction of Th1 cytokine in the CD4+ subset, while the frequency of IL-4+ CD4+ Th2 cells increased during co-culture of PBMCs with MSCs. MSC-treatment modulated the balance of Th1/Th2 in PBMCs from Th1-dominant to Th2-dominant.