Original Research Article

Preconditioning with IFN-γ and LPS improves the immunomodulatory potential of bone marrow-derived clonal mesenchymal stem cells

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DOI: 10.22034/HBB.2023.16

Received: May 31, 2023; **Accepted:** June 8, 2023

ABSTRACT

Mesenchymal Stem Cells (MSCs) are one of the promising cellular therapies for immune-related diseases. Preconditioning of MSCs increases their efficacy and overcomes several hurdles in MSC-based therapies. It reduces tissue inflammation and improves cell survival, consequently enhancing therapeutic outcomes. Evaluating some proteins may elucidate the probable behavior of transplanted cells in cellular therapies. Improving the number of immunomodulatory factors like Programmed cell death ligand 1 (PD-L1), Indoleamine 2,3-dioxygenase (IDO), and Tumor Necrosis Factor (TNF)-Stimulated Gene-6 (TSG-6) could be fundamental to modulate the immune system in immune-related disorders. In the current study, human Bone Marrow-derived clonal MSCs (hBM-cMSCs) were preconditioned with different regimens, especially Interferon-gamma (IFN-γ) to evaluate the effects of these regimens on the desired protein expression patterns to pave the way for clinical applications of MSCs according to their protein production potentials and enhance their therapeutic potential.

***Keywords*:** Immunomodulation, mesenchymal stem cell, preconditioning

INTRODUCTION

Mesenchymal Stem Cell (MSC) is an essential immunotherapeutic option with multiple potential applications for immunological-based disease therapies [1]. MSC is a multipotent, non-hematopoietic population of cells that can be obtained from various body tissues, such as bone marrow, placenta, and adipose tissue, making them widely available for regenerative and immunomodulatory applications [2,3]. MSCs own the potential to differentiate into trilineage cell types, which convert them into an ideal cellular therapy candidate for diverse diseases, especially immune-related diseases. MSCs can also be grown in the laboratory for large-scale production, which makes them a cost-effective solution for clinical use. MSCs have been shown to have immunomodulatory properties, which makes them a promising candidate for treating autoimmune diseases [4]. It has been shown that there are differences in the transcriptomic and proteomics profiles of MSCs, as well as differences in the miRNome and secretome [5]. Recent research suggests that MSCs do not possess immunosuppressive characteristics at the outset but should be stimulated to obtain these characteristics by environmental stimuli such as biomolecules (inflammatory cytokines) [6]. Priming or preconditioning of MSCs is an option to increase efficiency and overcome several hurdles in MSC-based therapies. For instance, preconditioning MSCs with cytokines and other growth factors is an effective way to improve their immunomodulatory potential and induce differentiation into therapeutic cell types. In addition, priming of MSCs has been used to increase their ability to survive after transplantation and to enhance the therapeutic effects [7]. In this regard, MSC preconditioning approaches have been evaluated for different applications, including cardiomyopathies, and degenerative and inflammatory diseases resulting in improved therapeutic outcomes [8]. These approaches have also been used to address central nervous system disorders, such as stroke [9]. MSC preconditioning has been investigated to reduce inflammation and increase cell survival, improving therapeutic outcomes. However, more experimental works are unavoidable to reveal the long-term effects of MSC preconditioning, as well as to understand its potential for treating other diseases [10]. MSC preconditioning with hypoxia involves exposing MSC to low oxygen levels, which can cause the cells to

release protective molecules that promote cell survival and reduce inflammation [11]. This is important because it can help to determine the most effective doses and the best ways to deliver the MSC preconditioning. Additionally, understanding the long-term effects of MSC preconditioning may help to identify potential risks associated with the treatment, as well as potential new applications for MSC preconditioning that could benefit patients in the future. MSCs that have been preconditioned with hypoxia are more resistant to the damaging effects of the body's immune system, making them more effective at treating inflammatory diseases. This means that MSCs that have been preconditioned with hypoxia may be able to provide longer-lasting relief from inflammation than unprimed MSCs [12].

Indoleamine-pyrrole 2,3-dioxygenase (IDO or INDO) is a factor that contains heme and is physiologically expressed in several tissues, including the small intestine, lungs, and the female genital tract. This protein is encoded by the IDO1 gene in humans and plays a vital role in the catabolism of the essential amino acid tryptophan [13]. It is involved in several physiological and pathological conditions, such as inflammation and immunomodulation. It is also involved in cancer pathogenesis and is a potential target for drug screening and drug development. IDO1 activity is also linked to the onset of autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis [14]. IDO plays a pivotal role in tolerance mechanisms, as well as in suppressing potentially destructive inflammatory processes in the body. It takes part in the maintenance of immune homeostasis and the suppression of autoimmune reactions. It also plays a regulatory role in inflammation and the immune responses to the cancer [15]. IDO is involved in natural defenses against microorganisms. The activity of IDO destroys pathogens by destroying tryptophan, which is essential for their survival. IDO is a critical component in the immune system, and its expression is modulated by pro-inflammatory cytokines (interferon-gamma), which is why its expression increases during inflammatory diseases and even in tumorigenesis.

Programmed death ligand 1 (PD-L1) is a protein encoded by the CD274 gene in humans. PD-L1 is a member of the B7 family of cell surface proteins and is expressed on monocytes, activated B cells, T cells, dendritic cells, and other cell types [16]. It plays a crucial role in T cell

activation and functions as an immune-checkpoint molecule, helping to prevent autoimmunity [17]. PD-L1 binds to a surface protein of activated T cells, called programmed cell death protein 1 (PD-1), and transmits an inhibitory signal to them, resulting in immune suppression [18]. This process contributes to maintains peripheral tolerance and helps keep the immune system from attacking healthy cells and tissues [19].

Tumor Necrosis Factor (TNF)-Stimulated Gene-6 (TSG-6) is a protein with anti-protective and inflammatory functions, including the ability to mediate many immunomodulatory and regenerative effects of mesenchymal stem cells [20]. TSG-6 also acts as a mediator of the immunomodulatory effects of mesenchymal stem cells-derived extracellular vesicles. TSG-6 inhibits the production of pro-inflammatory cytokines and chemokines and modulates the polarization of macrophages. High amounts of this protein is a valuable supplement for reducing chronic inflammation [21,22]. It also has strong antioxidant characteristics that protect cells from free radicals. This makes it a useful supplement for supporting overall health and wellness. Evaluating some proteins may estimate the probable behavior of transplanted cells or cellular byproducts in cell- or cell-free therapies. Enhancing the amount of immunomodulatory factors like IDO, PD-L1, and TSG-6 could be fundamental to modulating the immune system in immune-related disorders. Therefore, in the current research, human bone marrow-derived clonal MSCs were preconditioned with different treatment regimens, especially Interferon-gamma (IFN-γ) to investigate how these preconditionings affect the expression patterns of desired proteins to prepare the groundwork for the clinical applications of MSCs according to their production patterns of proteins. This study can be used to optimize MSC culturing protocols and maximize MSC potential in regenerative medicine. Additionally, our findings could be used to develop novel cell therapy strategies.

MATERIALS AND METHODS

Culture and expansion of clonal mesenchymal stem cells

Allogeneic human bone marrow-derived clonal mesenchymal stem cells from Royan Advanced Therapy Medicinal Product-Technology Development Center (Royan ATMP-TDC) were obtained as frozen vials [1]. The vials contained 1×10^6 cells/ml of

medium in passage nine and plating in 150 cm^2 flasks with 20 ml of complete culture medium that comprised of Dulbecco Modified Eagle Medium (DMEM; Gibco, Paisley, Scotland, UK) supplemented with 5% human Platelet lysate (hPL; Cell Tech Pharmed Co., Iran), 1 % GlutaMAX™ (Gibco, Paisley, Scotland, UK), 1 % MEM Non-Essential Amino Acids (NEAA; Gibco, Paisley, Scotland, UK), and 1 % Penicillin-Streptomycin (5,000 U/mL) (Pen-Strep; Thermofisher, Madison, WI, USA). The cells were then counted using a hemocytometer and cell viability was determined by trypan blue (Sigma-Aldrich, USA) staining.

Preconditioning of mesenchymal stem cells

When cMSCs were grown to 60–70 % confluency, preconditioning was carried out as follows: As a control group, the first group was incubated for 48 h under normal conditions (37 °C, 85 % humidified incubator containing 5 % CO_2, normoxia). Interferon-gamma (50 ng/ml) (abcam, USA) was administered to the second group and incubated for 48 hours (37 °C, 85 % humidified incubator, 5 % CO_2, Normoxia). The third and fourth groups were preconditioned with polyinosinic-polycytidylic acid (Poly (I:C); Sigma-Aldrich, Israel) at 42.22 µg/ml and Lipopolysaccharide (LPS; Sigma-Aldrich, Israel) at 1 µg/ml, respectively, and incubated for 48 hours (37 °C humidified incubator containing 5 % CO_2, Normoxia). The fifth group was subjected to hypoxia for 48 h (37 °C, 85 % humidified incubator containing 1 % O_2, 94 % N_2, and 5 % CO_2). All supernatants from all groups were removed, and cells were washed after 48 hours; finally, a fresh medium was added to allow cMSCs to secrete their secretions within three days. For further experiments, the supernatant (Conditioned Media (CM)) was pooled and stored at -80 °C. The supernatants were used to measure cytokine/growth factor concentrations. The protein contents of the supernatant were then analyzed using Enzyme-Linked Immunosorbent Assay (ELISA) kits. The preconditioned cMSCs were frozen in liquid nitrogen.

Total protein assay for conditioned medium of preconditioned mesenchymal stem cells

The amount of total protein was measured using a commercial Bradford protein assay kit purchased from DNAbioTech, Iran. The process relies on creating a complex between the Brilliant Blue G dye and the proteins present in the solution. This

complex is essential for the success of the method. The formation of the protein-dye complex results in a change in the maximum wavelength of dye absorption, from 465 nm (yellowish green) to 595 nm (blue). The absorbance at 570 nm of the solution in each well was read using a Microplate reader.

Protein extraction and western blotting assay

Western Blotting (WB) was carried out following the standard protocols [23]. After homogenizing the MSCs with non-denaturing lysis buffer (Abcam, Cambridge, MA), they were centrifuged for 20 min at 10,000 g and 4 °C, and their supernatants were used for further experiments. Following 5 min of boiling, serum lysates were subjected to SDS-PAGE, and the gel proteins were transferred to PVDF membranes manufactured by Bio-Rad Laboratories (CA, USA). The membranes were then blocked for one hour at room temperature with 5% bovine serum albumin (BSA; Sigma Aldrich, MO, USA). Following blocking, the membranes were incubated for one hour at room temperature with primary antibodies derived from rabbits, including Anti-IDO (Santacruz, USA), and Anti-TNFAIP6 (TSG-6; Elabscience Biotechnology Inc., USA). As a control, an anti-β Actin antibody (Anti-ACTIN, Santacruz, USA) was used. Detection of membrane-bound antibodies was accomplished by using a chemiluminescence detection system (ECL Advanced Kit; GE Healthcare Biosciences, Pittsburgh, Pennsylvania). The expression of proteins was quantified using the UN-SCAN-IT gel analysis software version 6.1 (Silk Scientific Inc., Orem, UT).

Enzyme-linked immunosorbent assay

According to the manufacturer's instructions, human IDO and PD-L1 concentrations were measured using DuoSet® ELISA kits (R&D Systems, USA). We used a Tecan microplate reader at 450 nm to measure absorbance after loading standards and supernatants into the wells. The samples and standards were tested in triplicate. At the time of analysis, cytokine and growth factor values were normalized based on the protein content of the supernatant.

Statistical analysis

Experimental procedures were performed at least three times, and the results are expressed as means ± Standard Deviations (SD). Qualitative results were presented as a frequency and a percentage, as opposed to

quantitative results expressed as a mean and standard deviation. ANOVA and Tukey's posthoc test were used when the distribution for quantitative variables was normal. Kruskal-Wallis was used when the distribution was not normal. In order to compare qualitative variables, the chi-square test was used. In order to analyze the data, SPSS software (version 22, Inc, Chicago, IL, USA) was used. The *p* values less than 0.05 were considered statistically significant ($p<0.05$). The graphs were created using GraphPad Prism (version 9.4.1, San Diego, CA, USA).

RESULTS

Mesenchymal stem cell preconditioning increased total protein amount

All preconditioning regimens were applied manually on different groups (The timeline of preconditioning on cMSCs is illustrated in Figure 1-A). Determining the protein concentration in the solution was carried out by using the Bradford protein assay, a simple and rapid procedure for assessing the total protein concentration. An essential concept in this procedure is the binding of proteins to coomassie dye in acidic conditions, resulting in a change in color from brown to blue. Consequently, the intensity of the blue complex is proportional to the protein concentration in the sample and can be easily determined by an ELISA plate reader at 595 nm (Bradford assay schematic illustration is shown in Figure 1-B). The total protein was measured in five preconditioned cMSC groups following the Kit manufacturer's protocol. It is demonstrated that the total protein amount is increased in three different preconditioned groups compared to the Non-treated group (Poly (I:C) vs. Non-treated (*p*-value=0.027), LPS vs. Non-treated (*p*-value=0.019), Hypoxia vs. Non-treated (*p*-value=0.013)). Total protein amount has been increased when different preconditionings were applied. The most protein content had detected when cMSCs were pretreated with Poly (I:C) (6.47 mg/ml). Hypoxia (5.63 mg/ml), LPS (5.30 mg/ml), and IFN-γ (5.23 mg/ml) showed more changes in protein content secretion, respectively (Bar plot is shown in Figure 1-C).

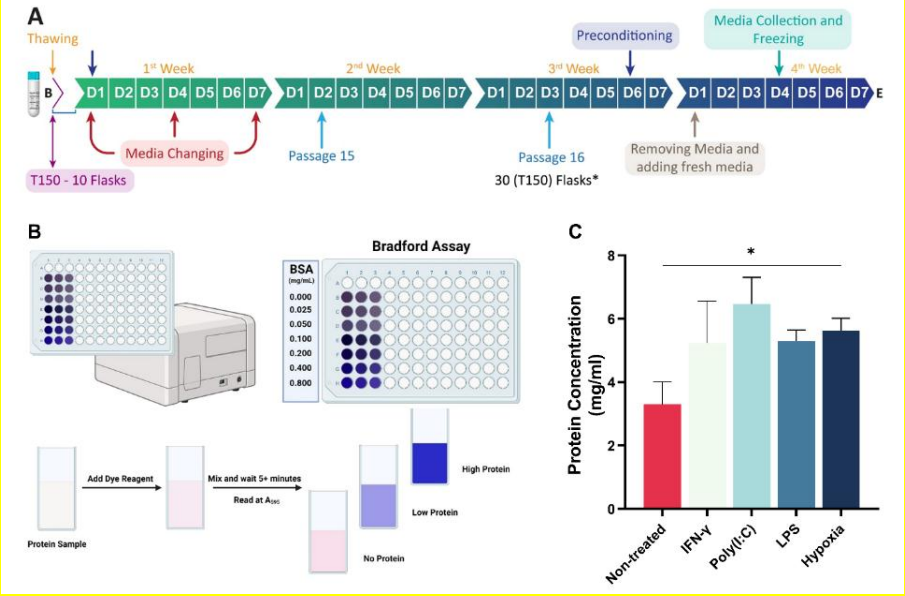

Figure 1. Timeline of Preconditioning of MSCs and Protein Content Measurements**: (A)** Timeline of preconditioning on MSCs; **(B)** Total protein content: Preconditioning resulted in an increase in protein concentration; **(C)** Bradford assay schematic illustration. Data is shown as mean ± standard deviation (SD) (n=3, *ns: not-significant, *: p<0.05*). *Abbreviations:* B: Begin; BSA: Bovine serum albumin; E: End; IFN-γ: Interferon gamma-treated group, LPS: Lipopolysaccharide-treated group, mg: Milligram; ml: Milliliter; MSC: Mesenchymal stromal cell, Poly (I:C): Polyinosinic:polycytidylic acid-treated group, SD: Standard deviation.

Mesenchymal stem cell preconditioning affected expression of TSG-6 and IDO

The expression of different proteins was assessed by using western blotting. Before pretreatments (The Non-treated group), protein expression had different patterns. However, applying different pretreatments on cultured cMSCs resulted in enhanced expression of TSG-6 protein in comparison with non-treated group. Aiming to consolidate this part of our study, IDO, and TSG-6, respectively, were evaluated by western blotting and normalized to β-Actin as a control. TSG-6 was affected by LPS more than other treatments (*p*-value=0.0006) (Figure 2-B and Figure 2-C). IDO expression was decreased in all pre-treatment groups except IFN-γ treated group in comparison with the Non-treated group. IDO was affected by IFN-γ more than other

treatments (*p*-value=0.0007) (Figure 2-B and Figure 2-C).

The LPS-treated group exhibits more production of anti-inflammatory factors (IDO and PD-L1)

Secretion of anti-inflammatory factor IDO was increased significantly in the IFN-γ-treated group (0.89±0.33 pg/mg), compared to the Non-treated group, which was 0.34±0.09 pg/mg (Figure 3) (*p*-value=0.0448). The highest IDO secretion was observed in the IFN-γ-treated group. On the contrary, secretion of PD-L1 as another anti-inflammatory factor was decreased significantly in the IFN-γ-treated group (27.52±20.90 pg/mg), compared to the Non-treated group, which was 247.74±48.90 pg/mg (*p*-value=0.002) (Figure 3). The highest PD-L1 secretion was observed in the Non-treated group. The LPS-treated group exhibits more production of anti-inflammatory factors (IDO and PD-L1).

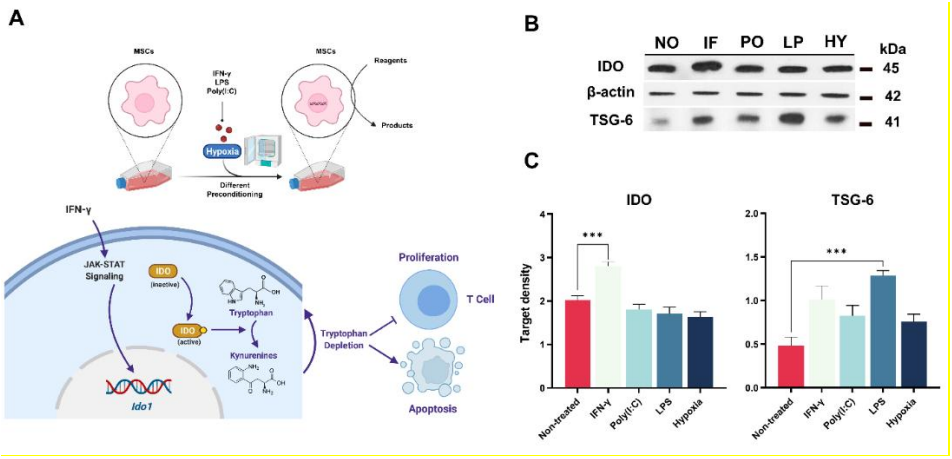

Figure 2. Western Blot Analysis Results of IDO and TSG-6: **(A)** Schematic illustration of IDO mechanism of action in immune suppression after preconditioning; **(B)** Representative WB of different preconditioned MSCs. β-actin was used as loading control; **(C)** Bar plots of western blot analysis of different preconditioned MSCs. Data are shown as mean ± standard deviation (SD) (n=3, *ns: not-significant, *: p<0.05, **: p<0.01, and ***: p<0.005*).
Abbreviations: HY: Hypoxia-treated group, IDO: Indoleamine 2,3-dioxygenase, IF: Interferon gamma-treated group, IFN-γ: Interferon gamma, JAK/STAT: Janus kinases/Signal transducer and activator of transcription proteins, kDa: KiloDalton, LP: LPS-treated group, LPA: Lymphocyte proliferation assay, µg: Microgram, MSC: Mesenchymal stromal cell, NO: Non-treated group, PO: Poly(I:C)-treated group, Poly(I:C): Polyinosinic:polycytidylic acid-treated group, SD: Standard deviation, TSG-6: Tumor necrosis factor (TNF) stimulated gene-6; WB: Western blotting

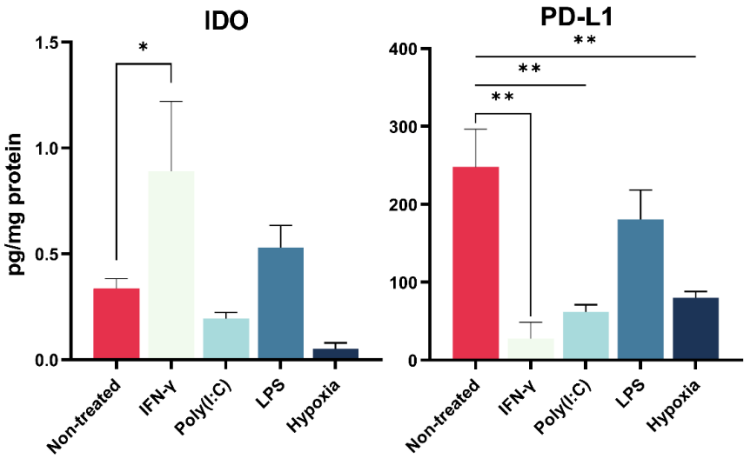

Figure 3. ELISA Results of IDO and TSG-6: Bar plots of Anti-inflammatory cytokines/growth factors including IDO and PD-L1 identified in CM of different preconditioned MSCs. Data is shown as mean ± Standard Deviation (SD) (n=3, *ns: not-significant, *: p<0.05, and **: p<0.01*).
Abbreviations: CM: Conditioned medium, ELISA: Enzyme-linked immunosorbent assay, IDO: Indoleamine 2,3-dioxygenase, IFN-γ: Interferon gamma-treated group, LPS: Lipopolysaccharide-treated group, mg: Milligram, pg: picogram, Poly(I:C): Polyinosinic:polycytidylic acid-treated group, SD: Standard deviation, TSG-6: Tumor necrosis factor (TNF) stimulated gene-6.

DISCUSSION

There is evidence of MSCs potential to modulate immune responses, reduce inflammation, and promote tissue repair. Therefore, MSCs may still be a promising potential therapy for immune-related diseases. For instance, in a research work on patients with Crohn's disease, treatment with MSCs showed a decrease in inflammation and an improvement in the patient's symptoms [24]. In another study, MSCs have been studied as a potential therapy for rheumatoid arthritis. Some promising results indicate they may reduce inflammation and improve disease symptoms [25,26]. Naïve MSCs grown in non-completed culture medium is the common part of current clinical trials, which do not contain immunomodulatory proteins prior the injection [27]. Essentially, the cells depend upon the patient's *in vivo* stimuli to develop an immunomodulatory phenotype. Therefore, this therapy serves as a suboptimal and unpredictable method, since the graft may not produce the same therapeutic effect from person to person. This lack of

reproducibility, efficacy, and predictability can limit the clinical success of this therapy, as well as its use in certain patient populations. In this regard, *in vitro* preconditioning of MSCs prior to transplantation has received more attention in recent years [16]. This can help to ensure that the cells will be in a better state to provide the therapeutic effects of the therapy and that the cells will be more likely to survive and thrive in the new environment with more adaptation. Preconditioning of different cell populations involves the application of various physical, chemical, or biological agents to the cells before their use in cell-based therapies. Furthermore, preconditioning can improve the therapeutic potential of desired cells by modulating the expression of specific genes. For instance, it has been demonstrated that *in vitro* preconditioning enhances MSC-CM effects by overexpressing cytoprotective genes. This allows for higher survival and proliferation of MSCs, as well as increased secretion of cytokines and growth factors. In addition, preconditioning can increase the anti-inflammatory properties of mesenchymal stem cells [28]. These increased levels of cytokines and growth factors can improve tissue repair and reduce inflammation, making cMSCs a promising tool for regenerative medicine. Our in vitro findings demonstrate that preconditioning of bone marrow-derived cMSCs significantly increases the expression of several factors and proteins in cMSCs and total protein concentration in CM derived from preconditioned cMSCs. *In vitro* preconditioning can also help to ensure that the therapeutic effect of therapy is more consistent and predictable across all patients [29]. Herein, we applied four preconditioning regimens on cMSCs and compared the effects of these preconditioning regimens on cMSCs in producing IDO, PD-L1, and TSG-6 as immunomodulatory factors. Notably, protein level was consistently superior in preconditioned cells compared to the Non-treated group. The most protein content had detected when cMSCs were pretreated with Poly (I:C). Hypoxia, LPS, and IFN-γ showed more changes in protein content secretion, respectively. This is likely because the preconditioning of the cells activates certain cellular pathways that stimulate the production of proteins [30]. Furthermore, this could be useful for various research and therapeutic applications. For example, preconditioned

cells could be used to create more efficient bioprocessing systems or to increase the production of therapeutic proteins in cell culture-based pharmaceuticals. However, it has been proven that IFN-γ is a key regulator of IDO expression and its role should not be underestimated. Increasing IDO levels is a natural feedback mechanism that controls excessive immune responses, which are produced both by tumor cells and macrophages [31]. IDO expression by cMSCs is a double-edged sword that has advantages in autoimmune disease but could be more challenging in tumor microenvironment to suppress immune effector cells. A study indicated that IFN-γ, TGF-β, and retinoic acid increased the expression of PD-L1 and the activity of IDO in mice models [32]. Our findings would seem to show that applying different preconditioning regimens on cultured cMSCs resulted in enhanced expression of TSG-6 protein in comparison with the Non-treated group. TSG-6 was affected by LPS more than other preconditioning regimens. IDO expression was decreased in all preconditioned groups except IFN-γ treated group in comparison with the Non-treated group. Our initial mechanistic assumptions were challenged by the protein-level findings. However, these results fail to explain why hypoxia alone could not induce any of these factors more than IFN-γ at the protein level. Based on ELISA studies, our findings showed that IDO protein showed the highest induction by IFN-γ, consistent with western blotting data and previous works. These results suggest that IFN-γ is an important regulator for IDO protein expression. This could have implications for immunological diseases and cancer research. Further studies should be conducted to understand how IFN-γ regulates IDO expression and how it can be used to target immunological diseases and cancer [33]. Hypoxia has previously been shown to upregulate IDO at the messenger RNA (mRNA) level and affect its post-transcriptional regulation in several ways [6]. On the contrary, secretion of PD-L1 as another anti-inflammatory factor was decreased significantly in the IFN-γ-treated group. The current study has provided further evidence that the highest PD-L1 secretion was observed in the Non-treated group; therefore, utilizing cMSCs with preconditionings could be a suitable option in cancer-associated research programs. The results point to the likelihood that the LPS-treated group exhibits more production of anti-inflammatory factors (IDO and PD-L1).

PD-L1 plays a pivotal role in the immunosuppressant effect of cMSCs on activated T-lymphocytes by reducing their proliferation and affecting their differentiation.

CONCLUSION

In brief, we have demonstrated that preconditioning of hBM-cMSCs resulted in a significant increase in several protein productions by the cells. Immunomodulatory protein expressions within the cells could be affected by preconditioning regimens, and IFN-γ, as well as LPS, played a crucial role in preconditioning of MSCs. IDO expression by MSCs is a double-edged sword that has advantages in autoimmune disease but could be more challenging in tumor microenvironments to suppress immune effector cells. In this regard, the current study suggests that preconditioning of MSCs is a promising option on the table to cope with difficulties using MSC in immune-related diseases through promoting their therapeutic potential.

ACKNOWLEGEMENT

This study was a part of the thesis submitted by Mehdi Soleymani-Goloujeh for a Ph.D. degree in the Faculty of Basic Sciences and Advanced Medical Technologies, Royan Institute, ACECR, Tehran, Iran. This project was financially supported by the Royan Institute for Stem Cell Biology and Technology (RI-SCBT, Grant No. 97000135). This project was supported by a grant provided by the Royan Institute for Stem Cell Biology and Technology (RI-SCBT, Grant No. 97000135). The authors wish to acknowledge the support they received from the Council for Development of Stem Cell Sciences and Technologies of the Iranian Vice-Presidency for Science and Technology (Grant No. 11/37646), Royan Stem Cell Technology Co. (RSCT, Grant No. 97040201), and the Iranian National Science Foundation (INSF, Grant No. 97012180).

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