

# Phenotypic and Functional Responses of Human Decidua Basalis Mesenchymal Stem/Stromal Cells to Lipopolysaccharide of Gram-Negative Bacteria

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**Introduction:** Human decidua basalis mesenchymal stem cells (DBMSCs) are potential therapeutics for the medication to cure inflammatory diseases, like atherosclerosis. The current study investigates the capacity of DBMSCs to stay alive and function in a harmful inflammatory environment induced by high levels of lipopolysaccharide (LPS).

**Methods:** DBMSCs were exposed to different levels of LPS, and their viability and functional responses (proliferation, adhesion, and migration) were examined. Furthermore, DBMSCs' expression of 84 genes associated with their functional activities in the presence of LPS was investigated.

**Results:** Results indicated that LPS had no significant effect on DBMSCs' adhesion, migration, and proliferation (24 h and 72 h) ( $p > 0.05$ ). However, DBMSCs' proliferation was significantly reduced at 10  $\mu\text{g/mL}$  of LPS at 48 h ( $p < 0.05$ ). In addition, inflammatory cytokines and receptors related to adhesion, proliferation, migration, and differentiation were significantly overexpressed when DBMSCs were treated with 10  $\mu\text{g/mL}$  of LPS ( $p < 0.05$ ).

**Conclusion:** These results indicated that DBMSCs maintained their functional activities (proliferation, adhesion, and migration) in the presence of LPS as there was no variation between the treated DBMSCs and the control group. This study will lay the foundation for future preclinical and clinical studies to confirm the appropriateness of DBMSCs as a potential medication to cure inflammatory diseases, like atherosclerosis.

**Keywords:** placenta, endothelial cells, proliferation, adhesion, migration, real-time PCR

## Introduction

Stem cell-based therapy and regenerative medicine have potentials to treat multiple diseases, including cardiovascular diseases (CVD), respiratory, neurological, skeletal, and autoimmune diseases. Moreover, what makes stem cells unique is their ability to release chemokines and receptors. These secretomes are needed for tissue regeneration, differentiation into various cell types, and self-renewal.<sup>1</sup> There are two classes of stem cells. They can be categorized to adult stem cells and embryonic stem cells.<sup>2</sup> Both have the capacity to differentiate to varied types of cells; therefore, they are suitable to use in regenerative medicine and cellular therapy.<sup>3</sup> Varied types of stem cells have been tested and evaluated, examples of which are embryonic stem cells (ESCs), bone marrow stem cells (BMSCs), induced pluripotent stem cells (iPSCs), hematopoietic stem cells (HSCs), neural stem cells (NSCs), very small embryonic-like stem cells (VSELs), mesenchymal stem cells (MSCs), and others.<sup>4-6</sup> Due to ethical controversies, applications of embryonic stem cells in clinical settings are restricted.<sup>6,7</sup>

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Nevertheless, mesenchymal stem cells (MSCs) gained attentions in medicine and were applied to stem cellular therapies because they have several advantages over other stem cells.<sup>1</sup> MSCs can directly differentiate when implanted in the damaged tissue.<sup>8–10</sup> MSCs regulate paracrine effects as they secrete cytokines and growth factors, thus increase inflammatory reaction or tissue fibrosis at the site of injury, which then stimulate endogenous progenitor cells and new blood vessels formation (angiogenesis).<sup>11,12</sup>

Moreover, at the site of injury, damaged tissues release micro-environmental factors that are related to ischemia gradients and inflammation; MSCs respond well to those factors released at the site of inflammation and migrate to the affected area.<sup>13–15</sup> Furthermore, MSCs can be found in various sources in the body including, bone marrow, muscles, umbilical cord, and other areas and have potential to differentiate and heal damaged tissues.<sup>16–18</sup> MSCs can be isolated from decidua basalis, which is a thin layer on the maternal tissue of the human placenta. These MSCs are called decidua basalis mesenchymal stem cells (DBMSCs).<sup>19,22</sup> They have several important functions in response to any injury and one of which is their capability to differentiate into numerous cell lineages, such as chondrocytes, adipocytes, and osteocytes.<sup>16,20</sup> DBMSCs are subjected to oxidative stress mediators and inflammation due to their significant location. They are located near the maternal circulation in their placental vascular microenvironment niche.<sup>21–23</sup> Recently, it has been shown that DBMSCs maintain their functions in oxidative stress environments induced by exposure to high levels of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).<sup>24</sup> In addition, DBMSCs can preserve endothelial cell functions from damage resulting from exposure to elevated levels of oxidative stress inducers, including H<sub>2</sub>O<sub>2</sub> and monocytes.<sup>23,25</sup>

Another form of oxidative stress or induced inflammatory microenvironment is the presence of endotoxins found on the outer membrane of gram-negative bacteria. Gram-negative organisms can be found in different locations on or in the human body. They are normally found in the gastrointestinal tract, genitourinary tract, and respiratory tract; and gram-negative aerobic and anaerobic bacteria colonize the human guts, which makes the human guts the largest reservoir of these microorganisms.<sup>26–29</sup> Endotoxin or lipopolysaccharide (LPS) has potential roles in the structural integrity of the gram-negative bacterial walls.<sup>30</sup> LPS is a complex glycolipid molecule.<sup>31,32</sup> The LPS aids these organisms by shielding them from environmental impacts as well as antimicrobial or

antibacterial products.<sup>30</sup> Microorganisms produce LPS not only in infections but can leak LPS through the healthy and intact mucosal lines of the guts.<sup>33–36</sup> Furthermore, the presence of harmful bacteria in the gut and LPS in the body is associated with several health conditions, such as inflammation, obesity, and metabolic disorder.<sup>31,32,37</sup> It is well known that persistent inflammation can potentially develop many diseases in the body, such as autoimmune diseases, arthritis, allergic reactions, cancer, and vascular diseases.<sup>35,38,39</sup> Continuous vascular inflammation can develop atherosclerosis, which is a chronic coronary heart disease, and potentially lead to health problems like cardiovascular diseases.<sup>39–41</sup>

Low concentrations of LPS can induce vascular inflammation and endothelial injury, which is characterized by dysfunction and phenotypic changes, including overexpression of inflammatory and adhesion molecules. Typical dysfunction includes increased endothelial cell proliferation and increased permeability, which is characterized by increased barrier functional activity of the endothelial cell layer allowing plasma macromolecules, such as lipoproteins to enter the intimal layer more easily.<sup>42–44</sup> LPS can be detected in the blood of healthy people at low concentrations ranging from 3 to 200 pg/mL.<sup>33–36</sup> This level of LPS in healthy individuals has the potential to activate endothelial cells and cause inflammatory responses, thus can lead to atherosclerosis.<sup>35,45–47</sup> Even if the individuals have healthy guts, low levels of LPS leak through the intact mucosa and end up in the splanchnic circulation.<sup>29,48,49</sup>

In this study, DBMSCs' functional responses in pro-inflammatory environments induced by LPS were evaluated. Several studies evaluated DBMSCs' responses to oxidative stress and found that under oxidative stress environment induced by H<sub>2</sub>O<sub>2</sub> as well as monocytes, DBMSCs' functional activities including adhesion, proliferation, and migration were maintained.<sup>23,25</sup> These significant results make DBMSCs as a suitable therapeutic target to repair endothelial cell injury and treat diseases caused by inflammation, such as atherosclerosis. Therefore, it was expected that DBMSCs would survive and function in harsh inflammatory environments induced by elevated levels of LPS.

In the current study, the main objective was to examine the ability of DBMSCs to survive in harsh inflammatory environments induced by high levels of LPS. Furthermore, the objectives were to study the consequences of LPS effects on DBMSCs' functional activities including adhesion, proliferation, and migration and to evaluate

DBMSCs' expression of genes associated with inflammation and their functional activities.

## Materials and Methods

### Ethics of Experimentation

This study was approved by the institutional research board (KAIMRC project # RC15/110/R & PNU Project # IRBC/246/13) at King Abdullah International Medical Research Centre (KAIMRC)/King Abdulaziz Medical City, Riyadh, Saudi Arabia. All placentae were obtained with informed consent from healthy patients. The study was conducted in accordance with the Declaration of Helsinki.

### Isolation and Culture of DBMSCs

MSCs have been isolated from the decidua basalis (DBMSCs) of human term placenta right after delivery, using a previously published method.<sup>19</sup> DBMSCs were cultured in a complete DBMSCs culture medium in flask T25 [DMEM-F12 medium containing 10% MSC FBS (mesenchymal stem cell certified fetal bovine serum, catalogue number 12-662-011, Life Technologies, Grand Island, USA), and antibiotics (100 µg/mL streptomycin and 100 U/mL penicillin)]. Incubation of the cells at 37 °C was performed in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air, in a cell culture incubator.

### Culture of DBMSCs with Different Concentrations of LPS

DBMSCs were cultured in varied concentrations (0, 0.50, 1, 10, 50, 100, 200 µg/mL) of LPS (Catalogue number TLRL-B5LPS, InvivoGen, USA). Experiments were completed in triplicate as well as repetition was performed independently with nine DBMSCs (passage 3) preparations.

### DBMSCs' Adhesion and Proliferation Assay Using the xCELLigence System

The xCELLigence system (RTCA-DP version; Roche Diagnostics, Mannheim, Germany) was used to evaluate the adhesion and proliferation of DBMSCs as this device automatically records and monitors real-time cell viability. The method used in this study was followed as previously published.<sup>23,25,50–52</sup> The experiments were initiated by addition of 100 µL of complete DBMSC growth medium in 16-well culture plates (Catalogue number 05469813001, E-Plate 16, Roche Diagnostics) in presence and absence of different concentrations (0, 0.50, 1, 10, 50, 100, 200 µg/mL)

of LPS. Plates were incubated at RT for 30 min then plates were placed in xCELLigence and instrument was set to blank. Plates were then removed from the machine. Next, the plates were seeded with  $1 \times 10^4$  DBMSCs in each well and were incubated for 30 min at RT in the biosafety cabinet. The culture plates were then placed in the xCELLigence system at 37°C in a cell culture incubator, and the DBMSCs' index was automatically monitored every 15 minutes for 72 h and analyzed through RTCA software [version 1.2.1.1002]; the data for cell adhesion was recorded at (2 h) and proliferation was recorded at (24, 48 and 72 h).<sup>23,25,50–52</sup> LPS treatments were changed every 12 hours. Each experiment has been done and repeated in triplicate.

### DBMSCs' Migration Assay Using xCELLigence System

DBMSCs' migration has been tested by applying the xCELLigence system, and CIM-16-well plates (Catalogue number 05665825001, CIM-16, Roche Diagnostics GmbH) as previously noted.<sup>23,25,50–52</sup> The migration experiments have been started by addition of growth medium with 10% 'fetal bovine serum (FBS) in the presence and absence of different concentrations (0, 0.50, 1, 10, 50, 100, 200 µg/mL) of LPS in the lower chamber and free serum medium has been added in the upper chamber. The CIM-plate 16 was placed in the incubator at 37°C for 1 hour then the plate was placed in xCELLigence and instrument was set to blank. Next, the CIM-plate 16 was seeded with  $1 \times 10^4$  DBMSCs in each well in the upper chamber and has been incubated for 30 min. Then, plate was placed in the xCELLigence system and DBMSCs migration has been monitored automatically every 15 min for 24 h using the xCELLigence system and RTCA software [version 1.2.1.1002]; then data have been presented as a cell index value.<sup>23,25,50–52</sup> Migration of DBMSCs with or without 10% FBS has been acted as a positive and negative control, respectively. Experiment has been done and repeated in triplicate.

### Gene Expression by Real-Time Polymerase Chain Reaction (RT-PCR)

DBMSCs' expression of RT<sup>2</sup> profiler PCR Arrays 84 genes related to Human Inflammatory Cytokines and Receptors (Catalog number PAHS-011Z, Qiagen, Hilden, Germany) has been detected as previously

published.<sup>19,23,24,52</sup> There were two DBMSC samples: the control samples (untreated) and samples that were treated with LPS. LPS treatments were changed every 12 hours during the 48-hours incubation period. After 48 h, the total RNA from DBMSCs [untreated DBMSCs, and DBMSCs treated with LPS] was extracted by using RNeasy Mini Kit (Qiagen, Hilden, Germany). Next, cDNA has been synthesized and utilized in a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Then, 20 µL of the mixture was added to each well in the RT<sup>2</sup> profiler PCR Arrays. The 96-well plate was then placed in the Thermal Cycle. The real-time polymerase chain reaction (RT-PCR) has been done in triplicate on the CFX96 real-time PCR detection system (BIO-RAD, California, USA). Then, the data were analyzed.<sup>19,23,24,52</sup> The  $\Delta\Delta^{-2}$  values have been then estimated to represent the findings as fold changes. The relative expression of internal controls (housekeeping genes) was used as provided in the RT<sup>2</sup> profiler PCR Arrays kit. GAPDH was used as a housekeeping gene for humans as provided in the kit. Trials were performed in triplicate using DBMSCs obtained from three placentae independently.

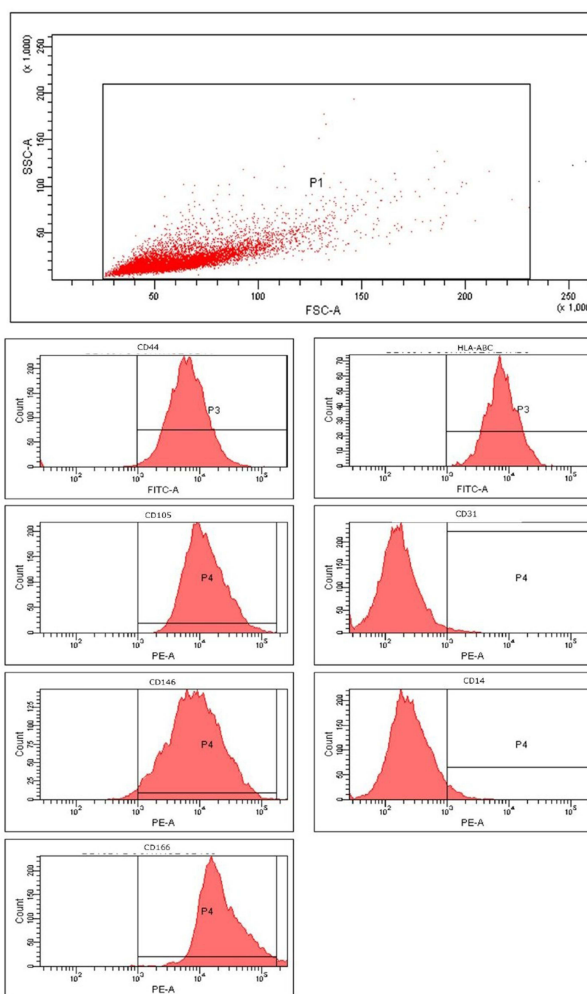
## Statistical Analysis

All the data shown in the bar graphs are presented as means  $\pm$  standard deviation (SD) of three independent experiments. To avoid bias, the experiments were performed three times independently. Using GraphPad Prism 9, we analyzed the data sets of two experimental groups using *t*-test, two tailed. Data deemed statistically significant if  $p < 0.05$ .

## Results

### Isolation and Culture of DBMSCs

MSCs from the decidua basalis (DBMSCs) were successfully isolated from human term placentas and cultured in complete DBMSCs DMEM/F12 supplemented with penicillin at 100 µg/mL and streptomycin at 100U/mL culture medium in a T75 cell culture flask, filter cap at 37°C in a cell culture incubator was previously described.<sup>25</sup> At passage 2, DBMSCs were positive for (>95%) MSC markers, and negative for haematopoietic markers (Figure 1). DBMSCs were able to differentiate into adipocytes, chondrocytes and osteocytes. These properties of DBMSCs were consistent with what we have reported previously.<sup>19</sup> In all experiments, DBMSCs were used at passage 2.

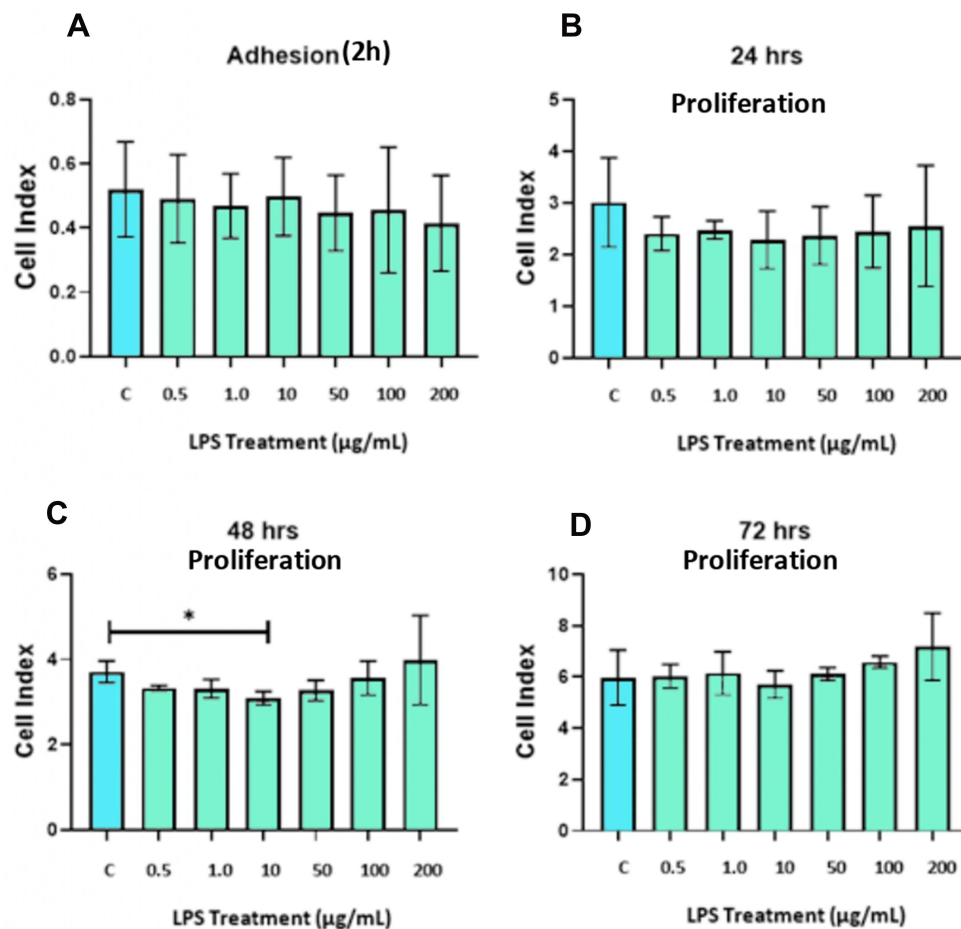


**Figure 1** Characterization of decidua basalis mesenchymal stem/stromal cells (DBMSCs) for mesenchymal stem cell (MSC) markers. Flow cytometric analysis of DBMSCs for MSC markers (CD44, CD105, CD146, CD166 and HLA-ABC); and MSC-negative markers (CD31 (endothelial cell) and CD14 (macrophage/monocyte marker)).

### LPS Dose Response and Time Course for Experimental Standardization

To assess the impact of LPS on DBMSCs' proliferation, DBMSCs have been cultured and treated at different concentrations of LPS and monitored for 72 h by using xCelligence system. After 24 h, the proliferation of treated DBMSCs with concentrations of 0.50, 1, 10, 50, 100, 200 µg/mL LPS showed no significant changes in comparison with the control ( $p > 0.05$ ) (Figure 2B). After 48 h, the proliferation of treated DBMSCs was significantly decreased at 10 µg/mL LPS ( $p < 0.05$ ) while had no significant changes at 0.5, 1, 50, 100 and 200 µg/mL LPS ( $p > 0.05$ ) compared with the untreated DBMSCs (Figure 2C). Lastly, after 72 h, the proliferation of treated DBMSCs with continuous exposure of LPS had no significant change in comparison with the untreated group





**Figure 2** (A) LPS effects on DBMSCs' adhesion in comparison with the control group at 2 h. (B–D) LPS effects on DBMSCs' proliferation in comparison with the control group at 24 h, 48 h, and 72 h. Experiments were performed three times independently, and the data are represented as mean  $\pm$  standard deviation. \*Significant ( $p < 0.05$ ).

( $p > 0.05$ ) (Figure 2D). Based on the results above, the exposure time of 48 h and 10  $\mu\text{g/mL}$  LPS was selected to evaluate the effects of LPS on the functions of DBMSCs (morphology, migration and gene analysis).

### LPS Effects on DBMSCs' Morphology

To see the effect of LPS on DBMSCs' morphology, DBMSCs were cultured in 10  $\mu\text{g/mL}$  LPS in complete DMEM/F12 for 48 h. Microscopic analysis showed no change in the morphology of 10  $\mu\text{g/mL}$  LPS treated DBMSCs in compared with the untreated DBMSCs at 24 and 48 h (Figure 3). The viability of DBMSCs treated with 10  $\mu\text{g/mL}$  LPS was  $>95\%$ .

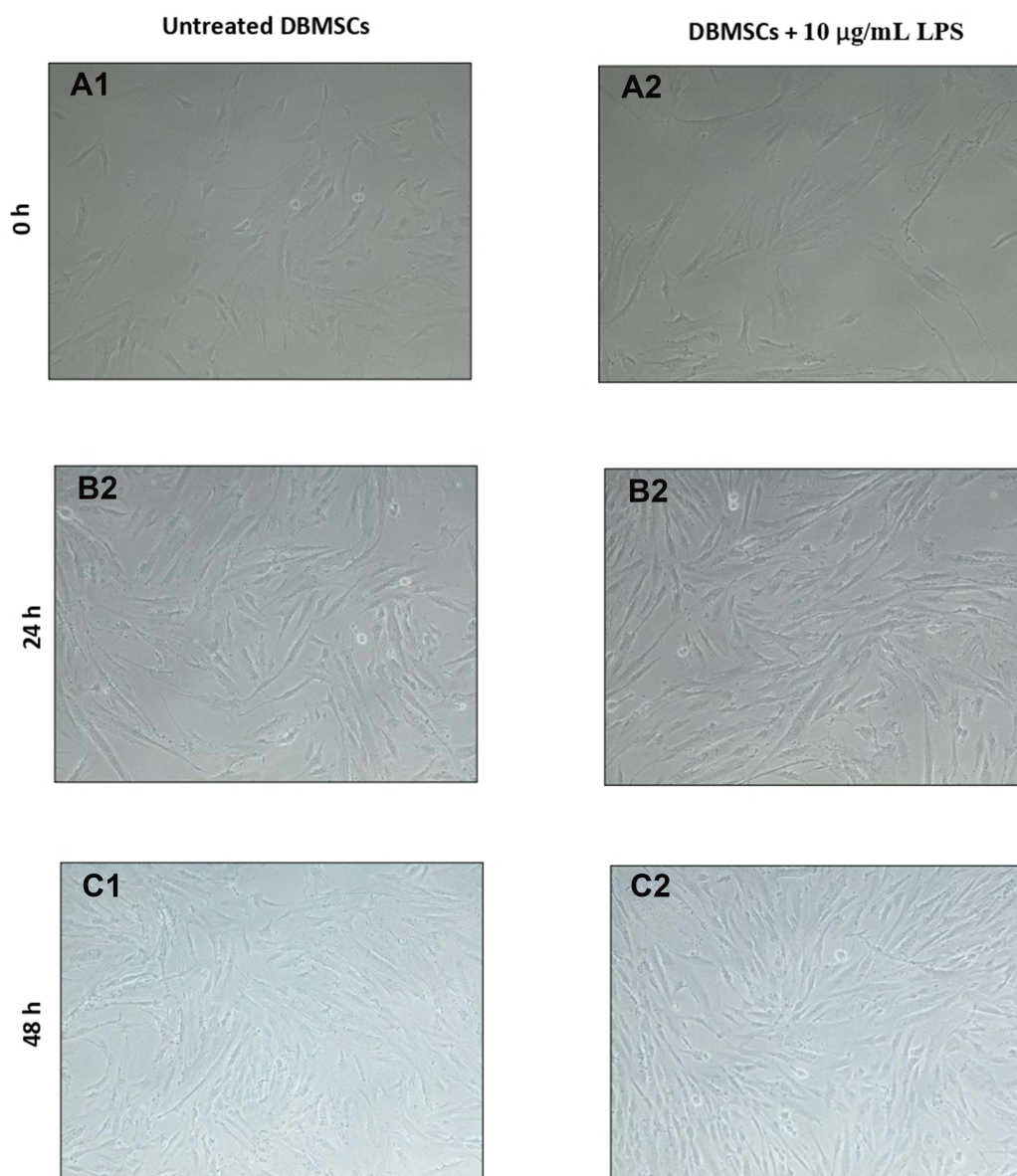
### LPS Effects on DBMSCs' Migration

Under continuous exposure to LPS, the migration of DBMSCs in the upper chamber of the migration plates was examined and monitored by the xCELLigence system

for 24 h. After 24 h, no significant impact of LPS on DBMSCs' migration ( $p > 0.05$ ) was stated compared to the control (Figure 4).

### LPS Modulates Various Gene Expression Related to Inflammatory Cytokines and Receptors in DBMSC

Total RNA was extracted from untreated and 10  $\mu\text{g/mL}$  LPS treated DBMSCs for 48 h. the gene expression of inflammatory cytokines and chemokines was analyzed and assessed using RT-PCR. Then the  $\Delta\Delta^{-2}$  values were used to yield the fold change values. In Tables 1–3, as well as Figure 5, results indicated that LPS influenced and modulated DBMSCs' expression of various genes that are related to human inflammatory cytokines and receptors. In Table 4 and Figure 6 results showed cytokines and receptors that mediate DBMSCs' functional responses, such as proliferation, migration, and differentiation were



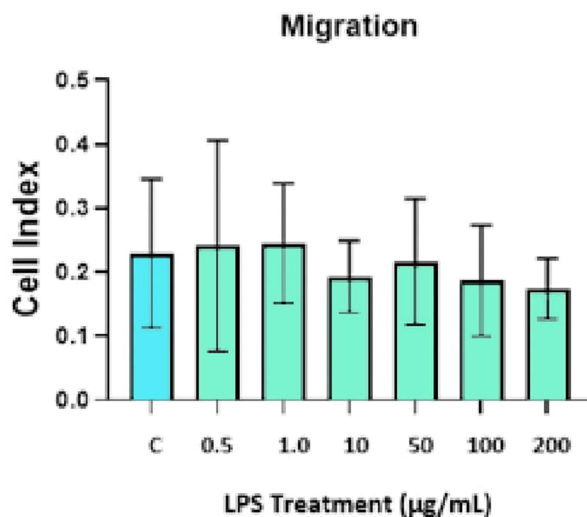
**Figure 3** (A1 and A2) Untreated DBMSCs vs DBMSCs treated with continuous exposure of 10 µg/mL LPS on the first hour when treatment was initiated at 60% confluency (100× magnification). (B1 and B2) Untreated DBMSCs vs DBMSCs treated with continuous exposure of 10 µg/mL LPS after 24 h of incubation period at 80% confluency (100× magnification). (C1 and C2) Untreated DBMSCs vs DBMSCs treated with continuous exposure of 10 µg/mL LPS after 48 h of incubation period at 90% confluency (100× magnification).

overexpressed ( $p < 0.05$ ). In Table 5 and Figure 7 genes that mediate DBMSCs' migration properties such as CXCR2 and CXCL12 were down regulated in the presence of LPS ( $p < 0.05$ ). There are other genes that were down regulated in the presence of LPS. However, they are not necessarily related to DBMSCs' functions (Table 5). The number of overexpressed genes that are related to DBMSCs' functional activities (proliferation, adhesion, and migration) were 31 genes ( $p < 0.05$ ) (Table 4). On the other hand, the number of downregulated genes were

33 genes, 31 of which are not necessarily related to DBMSCs' functions while two of the genes were related to DBMSCs' migration properties (CXCR2 and CXCL12) ( $p < 0.05$ ) (Table 5). Biological activities of the genes in the following Tables 1–5 were obtained from GeneBank via <https://www.ncbi.nlm.nih.gov/nucleotide/>.

## Discussion

Mesenchymal stem cells have gained a lot of attention for their therapeutic agent properties over the past decade.



**Figure 4** LPS effects on DBMSCs' migration in comparison with the control group at 24 h. Experiments were performed three times independently, and the data are represented as mean  $\pm$  standard deviation.

Moreover, MSCs have properties to regenerate and differentiate into mesodermal lineages (adipocytes, osteoblasts, and chondroblasts) as well as neuroectodermic lineages (neurons or alveolar endothelial cells).<sup>53–55</sup> In addition, MSCs could be differentiated into non-mesodermal lineages (eg, cardiomyocytes, hepatocytes, or epithelial cells).<sup>56–58</sup> MSCs could be originated from the bone marrow; however, they have been isolated from varied origins in humans, such as adipose tissue, hair follicles, umbilical cords, placentas, muscle, peripheral blood, amniotic fluid, and other organs, such as brain, liver, or lung.<sup>16,54,59,60</sup> Moreover, MSCs have different sources and their multilineage differentiations are not the only potentials that make MSCs special. MSCs have potential to migrate to sites of inflammation in which they stimulate tissue regeneration by releasing paracrine factors as well as differentiating and replacing damaged tissues or cells.<sup>61</sup> Not only MSCs have the capabilities to heal but they also are involved in paracrine signaling and cell–cell communication.<sup>62</sup> Furthermore, MSCs have the ability to interact with the host niche at the site of injury or inflammation and promote the following functions: MSCs can inhibit the immune system, promote cell survival, and/or form new blood vessels via angiogenesis by releasing factors, such as IL-6.<sup>61,63</sup>

There are several impacts that influence the successive implications of MSCs in clinical settings when cell therapy is introduced to the patients. As a result of these challenges, 99% of MSCs will die a few days after transplantations, thus resulting in unsuccessful applications of this

approach.<sup>64–73</sup> These challenges can result from MSCs isolation techniques that will result in uncontrolled cell quality, or invasive isolation processes as there are several ways and approaches to isolate MSCs from tissues, loss of MSCs potency, limited lifespan of MSCs, or loss of MSCs properties when cells are expanding or proliferating in vitro. Other challenges could be initiated when MSCs are transplanted in the recipients' tissues or organs, such as stressful condition (oxidative stress, inflammation, radiation, and chemotherapy).<sup>64–73</sup> Interestingly, MSCs that are derived and isolated from maternal decidua basalis tissue of human placenta have unique phenotypic characteristics and can withstand environments that are highly stressful because pregnancy is a highly stressful condition in which the decidua basalis tissue is exposed to high amounts of oxidative stress or inflammatory environment.<sup>22–24,74–76</sup>

Previously, H<sub>2</sub>O<sub>2</sub> and monocytes were introduced to DBMSCs, and results demonstrated that DBMSCs maintained their phenotypic properties and functions.<sup>23</sup> LPS is another form of inflammatory environment inducer and in this study, LPS was introduced to DBMSCs, and their phenotypic functions were monitored and analyzed.

According to the International Society for Cellular Therapy (ISCT), for MSCs to be used as a therapeutic agent, they must meet the minimal criteria for defining their multipotent function; one of which is the MSCs ability to adhere to plastic surfaces when grown in vitro.<sup>77</sup> In the current study, DBMSCs were treated with LPS without preconditioning. Results showed that after 2 h, LPS had no significant effect on DBMSCs' adhesion ( $p > 0.05$ ). Khatlani et al<sup>24</sup> studied the multipotent properties of DBMSCs when they were preconditioned with H<sub>2</sub>O<sub>2</sub>. The authors found that DBMSCs' adhesion in presence of H<sub>2</sub>O<sub>2</sub> was significantly increased when they were preconditioned with H<sub>2</sub>O<sub>2</sub> for 72 h ( $p < 0.05$ ) while DBMSCs' adhesion that were not H<sub>2</sub>O<sub>2</sub>-preconditioned remained unchanged ( $p > 0.05$ ). Same trend of observation regarding DBMSCs' adhesion when subjected to a stressful condition without preconditioning was noted. Previous studies found that preconditioned MSCs, such as DBMSCs and BMMSCs with oxidative stress mediators (eg, H<sub>2</sub>O<sub>2</sub>, glucose, hypoxia) enhanced the MSCs' performance.<sup>24,76,78</sup> Several studies claimed that the possible reason why MSCs' performances were enhanced when preconditioned with hypoxia or oxidative stress could be due to activation of paracrine or pro-angiogenic pathways, so they could survive and adapt to oxidative stress environments.<sup>78–82</sup>

**Table I** LPS Effects on DBMSCs' Gene Expression of Cytokines and Cytokine Receptors.

Gene	Gene Full Name	Control Mean $\Delta\Delta^{-2}$ Value	10µg/mL LPS Mean $\Delta\Delta^{-2}$ Value		Fold Change Control vs 10µg/mL LPS P<0.05	Biological Activity
A: Results of Fold Change That are >2.0 in Control vs 10 µg/mL LPS (p < 0.05)						
CCL17	Chemokine (C-C motif) ligand 17	1	18.32	4.20	↑*	Migration property
CCL23	Chemokine (C-C motif) ligand 23	1	5.10	2.35	↑	Migration property
CXCL10	Chemokine (C-X-C motif) ligand 10	1	8480.52	13.05	↑	Migration property
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	1	15.62	3.97	↑	Migration property
CXCL11	Chemokine (C-X-C motif) ligand 11	1	5.86	2.55	↑	Anti-inflammatory
CX3CR1	Chemokine (C-X3-C motif) receptor 1	1	590.76	9.21	↑	Migration property
CXCL2	Chemokine (C-X-C motif) ligand 2	1	4.15	2.05	↑	Migration property
B: Results of Fold Change That are <2.0 in Control vs 10 µg/mL LPS (p < 0.05)						
CCL15	Chemokine (C-C motif) ligand 15	1	1.23	0.29	↑*	
CCL24	Chemokine (C-C motif) ligand 24	1	1.08	0.11	↑	
CCL26	Chemokine (C-C motif) ligand 26	1	1.43	0.51	↑	
CCL7	Chemokine (C-C motif) ligand 7	1	1.24	0.31	↑	
CXCL3	Chemokine (C-X-C motif) ligand 3	1	2.06	1.05	↑	Migration property
CXCL5	Chemokine (C-X-C motif) ligand 5	1	1.21	0.27	↑	Migration property
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	1	1.16	0.21	↑	
CCR4	Chemokine (C-C motif) receptor 4	1	3.12	1.64	↑	Migration property
CCR5	Chemokine (C-C motif) receptor 5	1	1.06	0.08	↑	
CXCR1	Chemokine (C-X-C motif) receptor 1	1	1.09	0.12	↑	Migration property
CCL11	Chemokine (C-C motif) ligand 11	1	0.03	-5.08	↓*	Anti-inflammatory
CCL13	Chemokine (C-C motif) ligand 13	1	0.17	-2.52	↓	Immunoregulatory and inflammatory processes
CCL20	Chemokine (C-C motif) ligand 20	1	0.16	-2.62	↓	Immunoregulatory and inflammatory processes
CXCL12	Chemokine (C-X-C motif) ligand 12	1	0.11	-3.20	↓	Migration property
CXCL13	Chemokine (C-X-C motif) ligand 13	1	0.02	-5.47	↓	B lymphocyte chemoattractant
CXCL9	Chemokine (C-X-C motif) ligand 9	1	0.15	-2.77	↓	Immunoregulatory and inflammatory processes
CCR3	Chemokine (C-C motif) receptor 3	1	0.13	-2.94	↓	
CXCR2	Chemokine (C-X-C motif) receptor 2	1	0.11	-3.16	↓	Migration property

(Continued)



Table 1 (Continued).

Gene	Gene Full Name	Control Mean $\Delta\Delta^{-2}$ Value	10 $\mu$ g/mL LPS Mean $\Delta\Delta^{-2}$ Value		Fold Change Control vs 10 $\mu$ g/mL LPS $P < 0.05$	Biological Activity
<i>CXCL1</i>	Chemokine (C-X3-C motif) ligand 1	1	0.30	-1.75	↓	Inflammation
<i>CCR1</i>	Chemokine (C-C motif) receptor 1	1	0.55	-0.85	↓	Inflammatory responses
<i>CCR6</i>	Chemokine (C-C motif) receptor 6	1	0.42	-1.25	↓	
<i>CCR8</i>	Chemokine (C-C motif) receptor 8	1	0.90	-0.15	↓	

Notes: \* = Upregulation. \* = Downregulation.

DBMSCs were treated with 10  $\mu$ g/mL LPS ( $p < 0.05$ ) and compared to the untreated DBMSCs for 72 h.

Next, DBMSCs' ability to proliferate in the presence of an pro-inflammatory mediator, such as LPS is important to evaluate DBMSCs' potential use in cellular therapies. Current results showed that LPS had no significant effect on DBMSCs' proliferation during the 24 h and 72 h ( $p > 0.05$ ). However, at 48 h, LPS significantly reduced DBMSCs' proliferation at 10  $\mu$ g/mL LPS ( $p < 0.05$ ) while had no significant changes on DBMSCs' proliferation at 0.5, 1, 50, 100, and 20,010  $\mu$ g/mL LPS ( $p > 0.05$ ). Khatlani et al<sup>24</sup> reported a similar finding in which the authors evaluated the effect of H<sub>2</sub>O<sub>2</sub> on DBMSCs' proliferation. After 24 h and 48 h, 1, 5, 25, 50, 100, and 200  $\mu$ M of H<sub>2</sub>O<sub>2</sub> had no significant effect on DBMSCs' proliferation ( $p > 0.05$ ); however, DBMSCs' proliferation was significantly reduced when they were treated with higher concentration of H<sub>2</sub>O<sub>2</sub> (400 and 600  $\mu$ M) ( $p < 0.05$ ). A study conducted by Kurte et al<sup>83</sup> found that MSC phenotypes can change depending on how long they were exposed to LPS. It was found that MSCs exerted different phenotypes when they were exposed to LPS at 1 h than when they were exposed to LPS at 24 h and 48 h. The authors claimed that the molecular explanation behind this phenomenon is still unclear.

In addition, results showed that IL-9 was overexpressed when DBMSCs were treated with 10  $\mu$ g/mL of LPS. However, Durand et al<sup>84</sup> found that LPS had no significant changes on IL-9 secretion by BMSCs. The variations in IL-9 expression in the presence of LPS could be related to the different types of MSCs investigated and treatment conditions. IL-9 has many important functions; it modulates and regulates several hematopoietic cells, it prevents programmed cell death (apoptosis), and most importantly it stimulates cell proliferation.<sup>85</sup> In a study conducted by Sun et al,<sup>86</sup> the authors found that IL-9 played an important role in trophoblast cells angiogenesis; and reduction of IL-9 led to

reduction of angiogenesis in trophoblast cells. In addition, IL1-B is a pro-inflammatory cytokine that has a great effect on MSCs proliferative properties.<sup>87-89</sup> In current study, IL1-B was overexpressed when DBMSCs were treated with 10  $\mu$ g/mL LPS. This finding correlated with a study conducted by Cui et al<sup>90</sup> in which the authors found that several pro-inflammatory cytokines, such as IL1-B that were secreted by MSCs in the lungs were upregulated under exposure of chronic airway LPS. However, additional future mechanistic study is necessary to confirm this.

To yield a successful outcome for MSCs' clinical applications, MSCs need to be able to migrate to the sites of inflammation (target tissues).<sup>77</sup> Results showed that LPS had no significant effect on DBMSCs' migration ( $p > 0.05$ ) after 24-h incubation in the upper chamber of the migration plates. Similarly, Khatlani et al<sup>24</sup> found that DBMSCs' migration in the presence of H<sub>2</sub>O<sub>2</sub> had no significant effect ( $p > 0.05$ ) while preconditioning DBMSCs with H<sub>2</sub>O<sub>2</sub> yielded a significant increase in DBMSCs' migration ( $p < 0.05$ ). Chemoattractant signals can influence the rate of MSCs' migration to the damaged tissue; and MSCs' migration is mediated by many chemokine ligands and receptors, such as CXCL12, CCR4, and others. These chemokines and receptors have an important effect in MSCs' inflammatory responses and migration.<sup>91-96</sup> Hereby, when DBMSCs were treated with 10  $\mu$ g/mL of LPS, various chemokines and receptors, including CCL23, CXCL10, CXCL11, CX3CR1, CXCL2, CCR4, CXCL3, and CXCL5 were significantly overexpressed ( $p < 0.05$ ). Previous studies noted that DBMSCs alone expressed CC, CXC and various chemokines and receptors family members, such as CCL1, CCL2, CCL21 and others. Nevertheless, DBMSCs alone did not express certain chemokines and receptors, such as CXCR1 and CX3CR1.<sup>19</sup> Yet, in the presence of LPS, DBMSCs

**Table 2** LPS Effects on DBMSCs' Gene Expression of Interleukins and Interleukin Receptors.

Gene	Gene Full Name	Control Mean $\Delta\Delta^{-2}$ Value	10 $\mu$ g/mL LPS Mean $\Delta\Delta^{-2}$ Value	Fold Change Control vs 10 $\mu$ g/mL LPS $P < 0.05$	Biological Activity
A: Results of Fold Change That are $>2.0$ in Control vs 10 $\mu$ g/mL LPS ( $p < 0.05$ )					
<i>IL17A</i>	Interleukin 17A	1	175901.84	17.42	$\uparrow^*$ Pro-inflammatory
<i>IL1B</i>	Interleukin 1, beta	1	4.30	2.10	$\uparrow$ Pro-inflammatory
<i>IL9</i>	Interleukin 9	1	1659291.13	20.66	$\uparrow$ Pro-inflammatory
<i>IL1RI</i>	Interleukin 1 receptor, type I	1	496.33	8.96	$\uparrow$ Pro-inflammatory
B Results of Fold Change That are $<2.0$ in Control vs 10 $\mu$ g/mL LPS ( $p < 0.05$ )					
<i>IL8</i>	Interleukin 8	1	2.38	1.25	$\uparrow^*$ Pro-inflammatory
<i>IL1A</i>	Interleukin 1, alpha	1	1.22	0.29	$\uparrow$
<i>IL1RN</i>	Interleukin 1 receptor antagonist	1	1.05	0.08	$\uparrow$
<i>IL7</i>	Interleukin 7	1	1.88	0.91	$\uparrow$
<i>IL13</i>	Interleukin 13	1	0.13	-2.91	$\downarrow^*$ Anti-inflammatory
<i>iIL15</i>	Interleukin 15	1	0.12	-3.02	$\downarrow$ T and natural killer cell activation and proliferation
<i>IL27</i>	Interleukin 27	1	0.18	-2.44	$\downarrow$ Pro- and anti-inflammatory properties
<i>IL5</i>	Interleukin 5 (colony-stimulating factor, eosinophil)	1	0.12	-3.08	$\downarrow$ B cells and eosinophils growth and differentiation
<i>IL9R</i>	Interleukin 9 receptor	1	0.13	-2.94	$\downarrow$
<i>IL10RB</i>	Interleukin 10 receptor, beta	1	0.60	-0.75	$\downarrow$
<i>IL5RA</i>	Interleukin 5 receptor, alpha	1	0.75	-0.41	$\downarrow$

Notes: \* = Upregulation. \* = Downregulation.

DBMSCs were treated with 10  $\mu$ g/mL LPS ( $p < 0.05$ ) and compared to the untreated DBMSCs for 72 h.

significantly overexpressed CXCR1 and CX3CR1 ( $p < 0.05$ ). However, CXCL12 was downregulated in the presence of LPS, and this result is correlated to a study conducted by Wobus et al.<sup>97</sup> In their study, the authors tested CXCL12 secretion and expression by MSCs in breast cancer cells in comparison to non-tumorigenic breast epithelial cells. They found that CXCL12 was downregulated in the breast cancer cells while the control group had no effect of CXCL12 secretion. The authors explained that cancer cells paracrine factors or small molecules derived from cancer or tumor cells could have the possible effect on this observation.<sup>97</sup> In the current study, LPS could be the reason why CXCL12 was

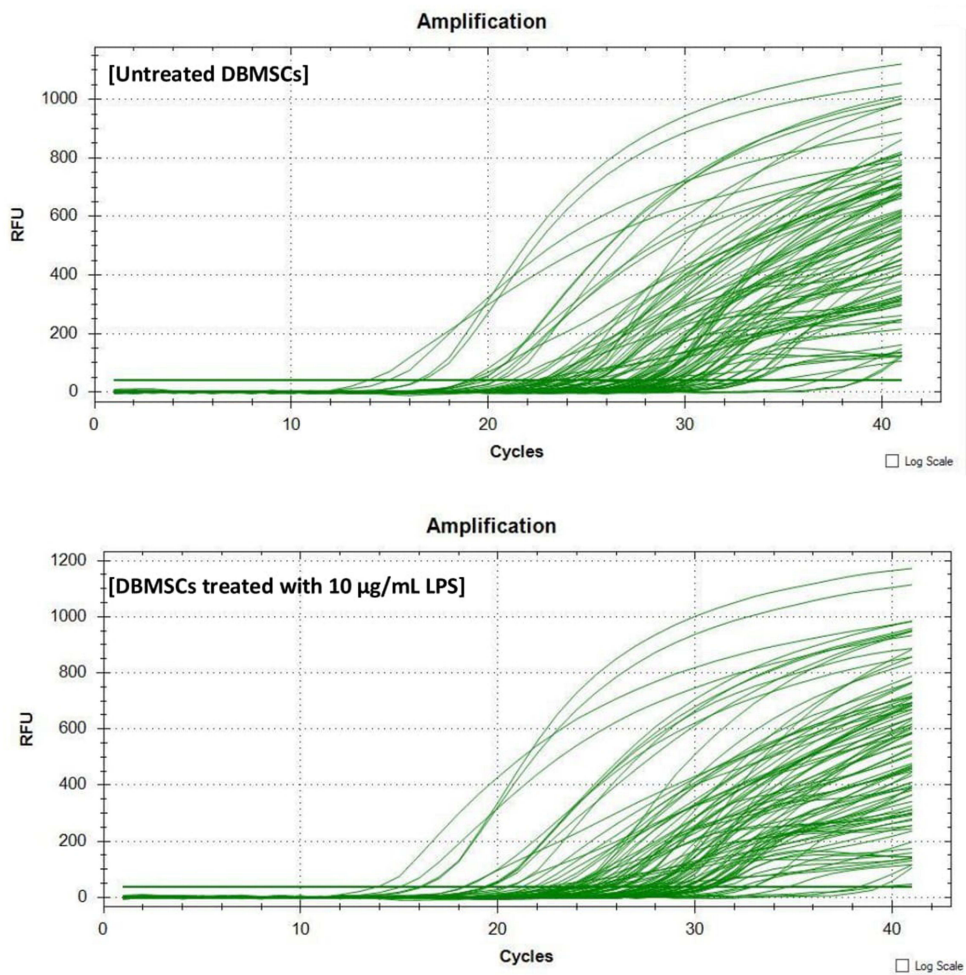
downregulated. It was found that overexpression of certain chemokine ligands or receptors, such as the chemokine receptor CXCR2, or chemokine ligands CXCL1, CXCL3, and CXCL5 can enhance MSCs' migration to the target tissue and promote cell survival.<sup>98</sup> In Shen et al study,<sup>98</sup> MSCs were exposed to radiation/chemical-induced oral mucositis and the authors found that CXCR2 level was overexpressed after radiation and was peaked at day 7 and eventually started to decline. In addition, CXCL1, CXCL3, and CXCL5 were upregulated after exposure to radiation. However, in the presence of LPS, CXCR2 was significantly downregulated. Different trends in oxidative stress inducers

**Table 3** LPS Effects on DBMSCs' Gene Expression of Other Cytokines and Other Cytokine Receptors.

Gene	Gene Full Name	Control Mean $\Delta\Delta^{-2}$ Value	10 $\mu$ g/mL LPS Mean $\Delta\Delta^{-2}$ Value	Fold Change Control vs 10 $\mu$ g/mL LPS $P<0.05$		Biological Activity
A: Results of Fold Change That are >2.0 in Control vs 10 $\mu$ g/mL LPS ( $p < 0.05$ )						
BMP2	Bone morphogenetic protein 2	1	8.72	3.12	$\uparrow^*$	Differentiation properties
B: Results of Fold Change That are <2.0 in Control vs 10 $\mu$ g/mL LPS ( $p < 0.05$ )						
CD40LG	CD40 ligand	1	1.43	0.51	$\uparrow^*$	
CSF2	Colony-stimulating factor 2 (granulocyte-macrophage)	1	2.03	1.02	$\uparrow$	
CSF3	Colony-stimulating factor 3 (granulocyte)	1	3.88	1.96	$\uparrow$	
TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4	1	1.09	0.13	$\uparrow$	
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	1	1.37	0.46	$\uparrow$	
AIMP1	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	1	0.65	-0.62	*	Inflammatory properties
CSF1	Colony-stimulating factor 1 (macrophage)	1	0.67	-0.58	$\downarrow$	Pro-inflammatory responses
IFNG	Interferon, gamma	1	0.87	-0.21	$\downarrow$	Immunoregulatory functions
LTB	Lymphotoxin beta (TNF superfamily, member 3)	1	0.40	-1.31	$\downarrow$	Inflammatory responses
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	1	0.74	-0.43	$\downarrow$	Immunoregulation and inflammation
NAMPT	Nicotinamide phosphoribosyltransferase	1	0.95	-0.07	$\downarrow$	Metabolism, stress response and aging
OSM	Oncostatin M	1	0.95	-0.07	$\downarrow$	Growth regulator
SPPI	Secreted phosphoprotein 1	1	0.44	-1.18	$\downarrow$	
TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	1	0.37	-1.45	$\downarrow$	Proliferation and differentiation of B cells
VEGFA	Vascular endothelial growth factor A	1	0.50	-1.00	$\downarrow$	
IFNA2	Interferon, alpha 2	1	0.10	-3.35	$\downarrow$	Antiviral, antiproliferative and immunomodulatory properties
FASLG	Fas ligand (TNF superfamily, member 6)	1	0.16	-2.63	$\downarrow$	
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	1	0.17	-2.56	$\downarrow$	Inflammatory, immunostimulatory, and antiviral responses
TNF	Tumor necrosis factor	1	0.16	-2.65	$\downarrow$	Pro-inflammatory properties

**Notes:** \* = Upregulation. \* = Downregulation.

DBMSCs were treated with 10  $\mu$ g/mL LPS ( $p < 0.05$ ) and compared to the untreated DBMSCs for 72 h.



**Figure 5** RT-PCR amplification curve of the untreated DBMSCs vs DBMSCs treated with 10 µg/mL LPS (generated by CFX-96 Real-Time System). Experiments were performed three times independently, and the data are represented as mean.

could be the reason why these results did not correlate. Yet, chemokine ligands, such as, CXCL3, and CXCL5 were significantly overexpressed in the current study. These

chemokines and receptors are important because they trigger factors at the site of inflammation; and with the aid of cell adhesion molecules, they contribute to the mobilization and

**Table 4** Overexpressed Cytokines and Receptors That are Responsible for DBMSC Functions (Migration, Proliferation, Differentiation) in the Presence of LPS

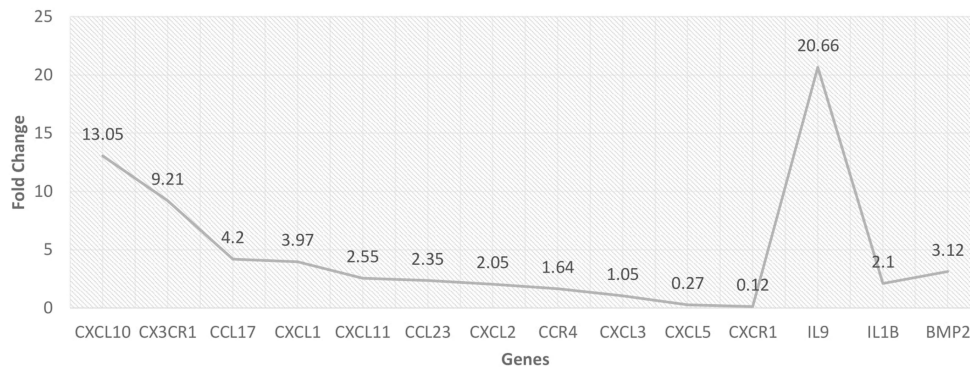
Gene	Fold Change Control vs 10µg/ mL LPS ( <i>p</i> <0.05)		Biological Activity	Gene	Fold Change Control vs 10µg/ mL LPS ( <i>p</i> <0.05)		Biological Activity
<i>CXCL10</i>	13.05	↑*	Migration	<i>CXCL5</i>	0.27	↑	Migration
<i>CX3CR1</i>	9.21	↑	Migration	<i>CXCR1</i>	0.12	↑	Migration
<i>CCL17</i>	4.2	↑	Migration	<i>IL9</i>	20.66	↑	Proliferation
<i>CXCL1</i>	3.97	↑	Migration	<i>IL1B</i>	2.1	↑	Proliferation
<i>CXCL11</i>	2.55	↑	Migration	<i>BMP2</i>	3.12	↑	Differentiation
<i>CCL23</i>	2.35	↑	Migration			↑	
<i>CXCL2</i>	2.05	↑	Migration			↑	
<i>CCR4</i>	1.64	↑	Migration			↑	
<i>CXCL3</i>	1.05	↑	Migration			↑	

**Note:** \* = Upregulation.



### Fold Change of Overexpressed Genes:

Control vs. DBMSCs +10 $\mu$ g/mL LPS  
( $p < 0.05$ )



**Figure 6** Overexpressed cytokines and receptors that are responsible for DBMSC functions (migration, proliferation, differentiation) in the presence of LPS. Experiments were performed three times independently, and the data are represented as mean.

migration of MSCs to the target tissues.<sup>91–96</sup> However, to investigate the mechanism of migration of DBMSCs in the presence of LPS will be investigated in a later study.

Upon MSCs' recruitment to the sites of the injury or the target tissues, MSCs secrete several mediators to initiate tissue repair and regeneration, such as anti-inflammatory, anti-apoptotic, immunomodulatory, and other agents. Both anti-inflammatory and immunomodulatory have a significant and main role in the therapeutic aspects of MSCs' application in clinical settings.<sup>99–101</sup> MSCs also modulate and regulate

soluble factors that are related to immune responses, such as PGE2, TGF- $\beta$ , IL-6, hepatocyte growth factor (HGF), or others.<sup>14,102–104</sup> Results showed that DBMSCs treated with 10  $\mu$ g/mL of LPS overexpressed certain interleukins and receptors. Du-Rocher et al found in their study through using chip array analysis that IL-17 signaling pathway is the second important pathways in MSCs.<sup>105</sup> Moreover, IL-17 is linked to other pro-inflammatory cytokines (IFN $\gamma$  and TNF $\alpha$ ) that promote the secretion of other chemokines, such as IL-6, IL-8, ICAM-1, and PGE2.<sup>106–109</sup> In their study, IL-

**Table 5** Downregulated Cytokines and Receptors That are Responsible for DBMSCs' Migration and Other Genes That are Not Necessarily Related to DBMSCs Functions in the Presence of LPS

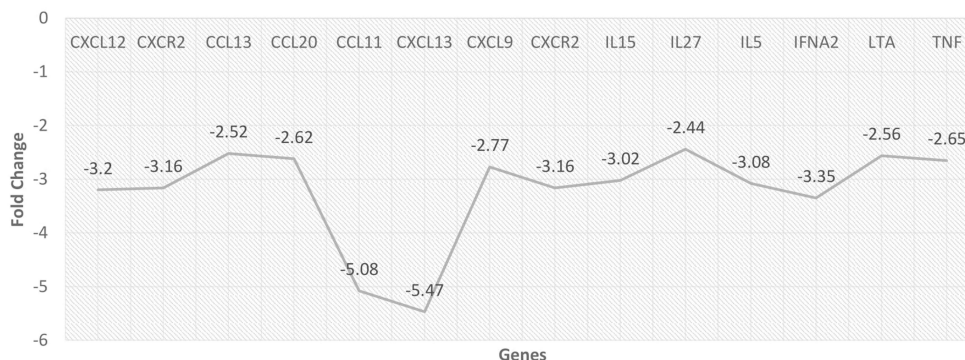
Gene	Fold Change		Biological Activity	Gene	Fold Change		Biological Activity
<i>CXCL12</i>	-3.2	↓*	Migration	<i>IL27</i>	-2.44	↓	Pro- and anti-inflammatory properties
<i>CXCR2</i>	-3.16	↓	Migration	<i>IL5</i>	-3.08	↓	B cells and eosinophils growth and differentiation
<i>CCL13</i>	-2.52	↓	Immunoregulatory and inflammatory processes	<i>IFNA2</i>	-3.35	↓	Antiviral, antiproliferative and immunomodulatory properties
<i>CCL20</i>	-2.62	↓	Immunoregulatory and inflammatory processes	<i>LTA</i>	-2.56	↓	Inflammatory, immunostimulatory, and antiviral responses
<i>CCL11</i>	-5.08	↓	Anti-inflammatory	<i>TNF</i>	-2.65	↓	Pro-inflammatory properties
<i>CXCL13</i>	-5.47	↓	B Lymphocyte Chemoattractant			↓	
<i>CXCL9</i>	-2.77	↓	Immunoregulatory and inflammatory processes			↓	
<i>IL15</i>	-3.02	↓	T and natural killer cell activation and proliferation			↓	

**Note:** \* = Downregulation.

## Fold Change of Downregulated Genes:

Control vs. DBMSCs +10 $\mu$ g/mL LPS

( $p < 0.05$ )



**Figure 7** Downregulated cytokines and receptors that are responsible of DBMSCs migration and other genes that are not necessarily related to DBMSC functions in the presence of LPS. Experiments were performed three times independently, and the data are represented as mean.

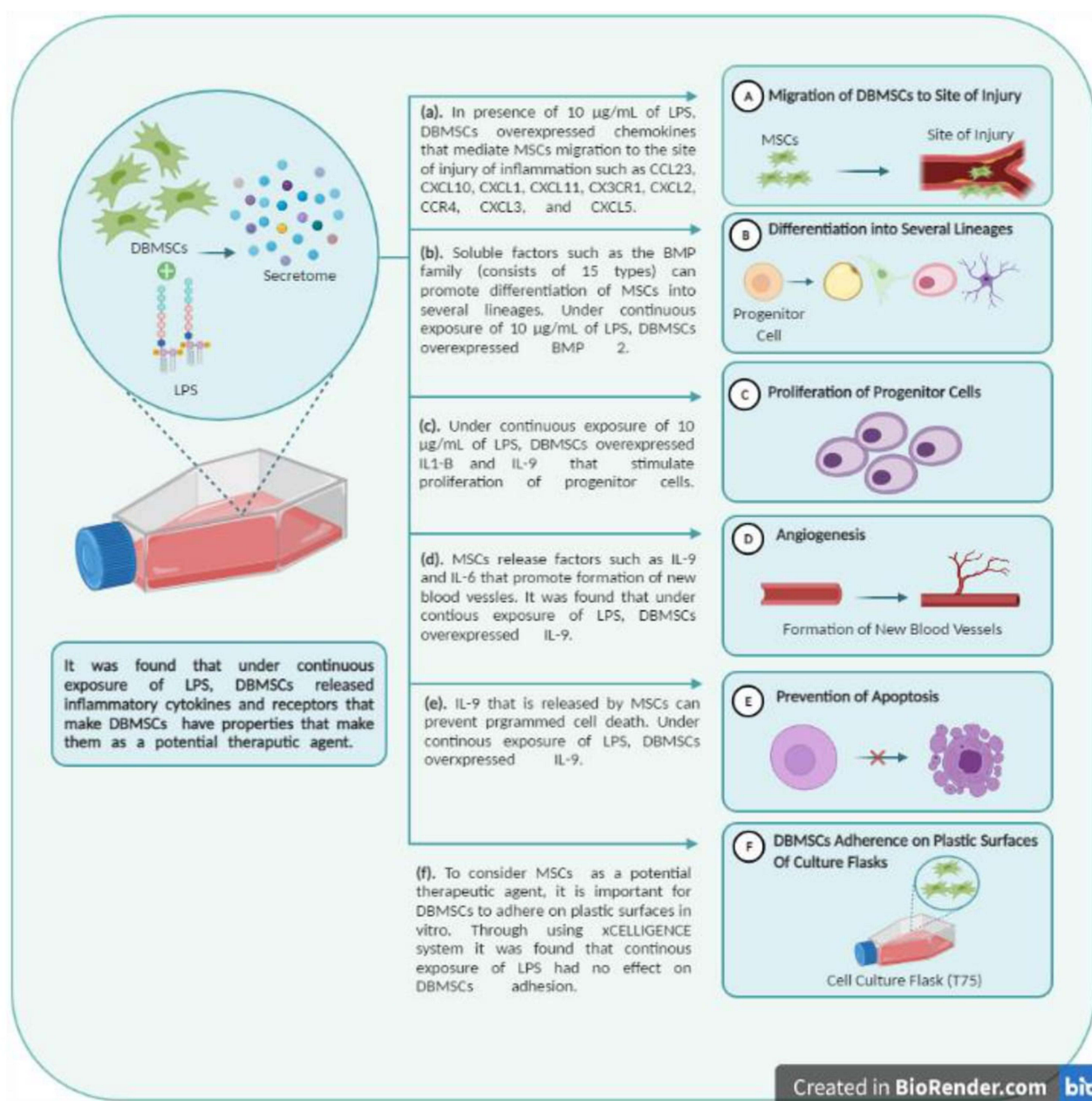
17 yielded large amounts of IL-8 and IL-6.<sup>105</sup> In the current study, IL-17A was overexpressed when DBMSCs were treated with 10  $\mu$ g/mL of LPS. IL-8 was also overexpressed in the presence of LPS. These findings correlate to a study conducted by Durand et al.<sup>84</sup> The authors evaluated the effect of certain factors, such as LPS on BMSC secretomes and they found that BMSCs secretory capacity was increased in the presence of LPS, such as IL-17A and IL-8.<sup>84</sup> Other soluble factors were overexpressed when DBMSCs were under continual exposure of 10  $\mu$ g/mL LPS, such as BMP-2. Our result correlated to a study in which the authors tested the effect of exosomes derived from human LPS-activated monocytes on MSCs osteogenic gene expression and found that BMP-2 was significantly increased.<sup>110</sup> The BMP family which consists of 15 members can promote MSCs' differentiation into several lineages and that depends on the micro-environment concentrations. Each member of BMP family will promote MSCs' differentiation to certain lineages. For instance, BMP-2, BMP-4, and BMP-7 can promote MSCs' differentiation to adipogenic lineage. BMP-2, BMP-6, and BMP-9 can modulate MSCs' differentiation to osteogenic lineage. Lastly, BMP-2 and BMP-7 can promote MSCs to differentiate into chondrogenic lineage.<sup>111,112</sup> The functional aspect of immune modulation of DBMSCs preconditioned with LPS will be determined in a future study.

These results and findings were in accordance with several studies on MSCs' functional responses and gene expression of cytokines and receptors when treated with several oxidative stress inducers. These results are evident

for the potential use of DBMSCs as a therapeutic agent for the treatment of inflammatory diseases, such as atherosclerosis since DBMSCs survived in harsh environments that were induced by high levels of LPS.

## Conclusion

In conclusion, DBMSCs have potential functional properties that enhance their application in cell-based therapy because of their abilities to work in highly oxidative stress environments as they maintained their functional activities when treated with H<sub>2</sub>O<sub>2</sub> and monocytes.<sup>23</sup> LPS is another form of inflammatory environment condition. Results of this study indicated that DBMSCs' functions (adherence, proliferation, and migration) were not significantly affected ( $p > 0.05$ ) when DBMSCs were treated with continuous exposures of different concentrations (0.50, 1, 10, 50, 100, 200  $\mu$ g/mL) of LPS (Figure 8). No variation between treated DBMSCs and the control group indicated that DBMSCs can survive in pro-inflammatory condition, in the presence of LPS, and can maintain their functional activities (adherence, proliferation, and migration). Moreover, many chemokines and receptors that are related to DBMSCs' phenotypic properties, such as adherence, proliferation, differentiation, and migration were overexpressed when DBMSCs have been subjected to 10  $\mu$ g/mL of LPS. Nevertheless, future in vitro and in vivo studies on DBMSCs are necessary to assess the potential use of DBMSCs in treating inflammatory diseases. For instance, DBMSCs' ability to invade endothelial tissues or layers in LPS condition and time-



**Figure 8** The effect of LPS on DBMSCs' functional responses (proliferation, adhesion, and migration) (Created with BioRender.com).

related phenotype changes of DBMSCs in the presence of continuous exposure to LPS. Evaluating the functional responses of DBMSCs when preconditioned with LPS will be investigated in future studies.

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## Disclosure

The authors report no conflicts of interest in this work.

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