ORIGINAL ARTICLE

# Human Mesenchymal Stem Cells Impair the Proliferation of Monocytes Through Cell Cycle Interference

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## ABSTRACT

**Introduction:** Monocytes are essential phagocytic cells of the innate immune system as they are required for the maintenance of tissue homeostasis. However, accumulation of monocytes is implicated in various chronic inflammatory diseases like coronary heart disease, atherosclerosis and in autoimmune disorders. Therefore, the number of monocytes must be carefully regulated to avoid monocyte induced inflammatory disorders. Mesenchymal stem cells (MSCs) have shown to be effective against various inflammatory diseases due to their immunosuppressive properties. The present study was designed to evaluate the less understood immunomodulatory effect of MSCs on monocyte proliferation and survival. **Method:** Primary monocytes were isolated from peripheral human blood using CD14+ monocyte isolation kit. The in house produced umbilical cord MSCs were co-cultured with monocytes at different ratio and time; assessed for the monocyte viability, proliferation and cell cycle. **Results:** Mesenchymal stem cells suppressed monocyte proliferation in a dose-dependent manner. The antiproliferative effect of MSCs was mediated by cell cycle arrest, whereby monocytes were arrested in the G0/G1 phase of the cell cycle by preventing them from progress into S and G2/M phases. Although cell cycle arrest could potentially lead to apoptosis; however, MSCs significantly enhanced the monocytes survival and inhibited apoptosis. **Conclusion:** Human MSCs inhibit the stimulated monocyte proliferation without inducing cellular apoptosis at in vitro. These results reveal that MSCs can be utilised to control monocytes' quantity during an unwanted immune response to maintain homeostasis.

**Keywords:** Monocytes, Mesenchymal stem cells, Proliferation, Cell cycle, Immunomodulation

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## INTRODUCTION

Monocytes and macrophages are part of the innate immune system. Monocytes are one of the key regulators of immune responses, essential for the development of beneficial immune reactions during inflammation and infections (1, 2). Upon stimulation, monocytes migrate from the peripheral blood circulation to the site of infection in the form of macrophages. Chemokines and cytokines secreted by immune cells such as macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor GM-CSF), and interleukin-3 (IL-3) increase the production of monocytes in the bone marrow and prolong the survival of monocytes/macrophages (3-5). In a healthy organism, the innate immune system provides the first line of defence against pathogens. The protective inflammatory response develops according to the time scale, through different phases, from initiation, full inflammation, to resolution and re-establishment of tissue integrity (6). Hence inflammation is a very natural process of tissue response in order to reach equilibrium in restoring homeostasis (6). However, prolonged inflammation in a disease state displaces from homeostasis. Therefore, the chronic inflammation needs to be tightly controlled to avoid excessive tissue damages.

In recent years, cell-based therapies using mesenchymal stem cells (MSCs) for immune-mediated diseases such as graft-vs-host disease and autoimmune diseases have emerged as a promising way to control highly inflamed immune response (7). The presence of MSCs is broadly witnessed in almost all adult tissues; they maintain a constant stem cell pool via self renewal and differentiate into cartilage, bone, and fat (8, 9). The successful transplantation of autologous and allogeneic MSCs in various conditions such as myocardial infarction, corneal damage and lung injury in animal and humans have resulted partly from its anti-inflammatory properties

(10-12). At the same time, by possessing an inherent immunosuppressive activity, MSCs are able to home the damaged tissues/organs and subsequently mediate the tissue repair through a direct differentiation of host cells or enhance the repair process by secreting growth cytokines (13, 14). The immunomodulatory activity of MSCs affects almost all type of immune cells, including the innate and adaptive immune cells (15-17). The immunosuppressive functions of MSCs are delivered through a direct cell-to-cell contact or secetion of biomolecules that act through paracrine/autocrine manner. It has been exposed that the immunosuppressive functions of MSCs affect various phases of immune response, namely activation, proliferation and effector functions (3). In regards to MSCs-monocytes communication, the physiological interaction between MSCs and monocytes in bone marrow is still elusive. In the bone marrow, MSCs are believed to reside in the periosteal region of bone marrow, where it provides the necessary signals to the haematopoietic stem cells and progenitors to be in a quiescent state or differentiate into mature cells (18).

Monocytes are implicated in various diseases, and they play a significant role in the formation and progression of atherosclerosis. It has been reported that an increased number of circulating monocytes are involved in atherosclerosis in diabetic Akita mice (2). In this perspective, MSCs can be utilised as a tool to suppress inflammation by inhibiting monocyte proliferation. Much other research works documented the influence of MSCs in monocytes functions such as differentiation toward macrophages and dendritic cells, chemotaxis, phagocytosis and microbial killing ability (19-23). However, no data available exploring the impact of MSCs on monocyte proliferation. Thus, the current study aimed to decipher the effect of MSCs on monocyte proliferation. Although, monocytes are naturally non-proliferating cells in vivo and the bone marrow release of monocytes governs the number of monocytes, yet at in vitro culture, monocytes are able to expand upon stimulation. In the present study, the immunomodulatory activity of human umbilical cord-derived MSCs was tested specifically on the expansion of isolated human monocytes. The propagation or proliferation of monocytes is profoundly affected by cell cycle and apoptosis, whereby the output of MSCs on these two cellular processes was assessed in order to justify the impact of MSCs on cell proliferation.

## MATERIALS AND METHODS

### Mesenchymal stem cells cultures

Fully characterised human umbilical cord MSC (UC-MSC) were obtained from Stem cell & Immunity Research Group, Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, University Putra Malaysia (24, 25). Briefly, human samples were collected after obtaining written consent from the donors, and the use of the human samples was approved by the Ethics and Research Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Mesenchymal stem cells were cultured in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12) consisting of GLUTAMAX (Gibco, United Kingdom) and supplemented with 10% commercially available optimised MSC serum (Thermo Fisher Scientific, USA), 1% penicillin/streptomycin, 0.5% fungizone and 0.1% gentamycin (Gibco, United Kingdom). Early passages of MSCs (P3-P8) were used in all experiments. The in house produced MSCs were characterised according to the minimal criteria defined by the International Society for Cellular Therapy (ISCT) (data not shown) (25, 26).

### Isolation of human monocytes

Twenty-millilitre of whole blood was collected from a healthy donor with informed consent and immediately processed within 20 minutes by diluting in 1 x phosphate buffer saline (PBS) (Gibco, UK) without calcium and magnesium ions at the ration of 1:1. Diluted blood was layered over 5 mL ficoll-paque solution (GE Health care, Life Sciences, Sweden) for gradient centrifugation for 30 minutes at 1200 rpm without deceleration. The peripheral blood mononuclear cells (PBMC), which appeared as a white ring at the interface of plasma and ficoll-paque reagent was collected and immediately suspended in 25 mL of 1 x PBS buffer for monocyte separation. Cells were subjected for monocyte isolation according to the manufactures instructions using a commercially available Monocyte Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, human monocytes were isolated by depletion of non-monocytes (negative selection). Non-monocytes were magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies, as a primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to microbeads, as a secondary labelling reagent. The magnetically labelled non-monocytes were depleted by retaining them on a MACS® Column in the magnetic field of a MACS separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), while the unlabelled monocytes passed through the column. The purity of monocytes and the efficiency of the isolation method were assessed by $CD14^+$ expression of negatively sorted monocytes using flow cytometer. The monocyte isolation method was considerably efficient and robust as the percentage or purity of isolated $CD14^+$ cells was notably high, 90 ± 5.95 (data not shown).

### Proliferation assay

The $^3$H-thymidine assay was conducted to assess the proliferation of $CD14^+$ monocyte cells and also to observe the effect of MSCs on monocyte proliferation. Optimised monocyte proliferation setting was used for co-culture experiments (data not shown). Freshly isolated monocytes ($5x10^4$ cells/well), were cultured in a 96-well plate and were then stimulated with and without 100 µg GM-CSF/ IL-3 (Sigma Aldrich, Germany). MSCs were

seeded in reducing order with the dilution of 5x, 10x, 50x and 100x; incubated overnight. While monocytes were added to the preplated MSCs on following with a fixed number of cells ($5x10^4$ cells/well). The co-culture of MSCs and monocytes was performed using a direct co-culture system where monocytes were cultured on the surface of pre-plated MSCs. To confirm whether the secreted factors of MSCs in the MSCs conditioned media could confer an inhibitory activity on monocytes proliferation, supernatant (SN) of MSCs culture was consumed instead of the MSCs. The final ratio of monocytes to MSCs were 1:0.2, 1:0.1, 1:0.02 and 1:0.01. The culture was incubated for seven days, and pulsed with 10 µl ($^3$H-TdR) (Sigma Aldrich, Germany) at the final 18 hours before the measurement. Cells were harvested onto glass filter mat by using a 96-well plate automated cell harvester (Harvester Mach III M, TOMTEC, USA). The filter mat was dried using the oven (40°C) for 10 minutes before adding 5 mL of scintillation fluid (OptiPhase SuperMix Cocktail; Perkin Elmer, Boston USA). The filter mat was then sealed and fitted into a scintillation cassette for radioactive measurement using luminescent Microbeta counter (Perkin Elmer, Boston USA). Results were expressed as counts per minute (CPM).

### Cell cycle assay

To further explore the monocytes proliferation, cell cycle assay was performed to assess the fractions of cells at different phases of cell cycle such as $G_0/G_1$, S and $G_2$/M during cell division. Monocytes ($3 \times 10^5$ cells/well) were co-cultured in a 6 well plate in the presence or absence of MSCs at ration (1:0.1) ($3 \times 10^4$ MSC/well). Monocytes were stimulated with 100 µg of cytokines IL-3 and GM-CSF for 3, 5 and 7 days. At the end of each time point, cells were washed and fixed in 70% ethanol (Merck, USA) overnight. For staining, the fixed cells were washed twice with 1 x PBS containing 1% bovine serum albumin (BSA) (Amresco, UK) and incubated for 30 minutes in 25 µl/mL RNase (BD, USA), 50 µg/mL PI (BD, USA) and 450 µl 1x PBS with 1% BSA in the dark at RT. After 30 minutes incubation, $2 \times 10^4$ cells were acquired in LSR Fortessa flow cytometer and analysed using the FACS Diva software.

### Apoptosis assay

Apoptosis assay was performed using the Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson, Sandiego). Monocytes were cultured in a six-well plate ($3 \times 10^5$ monocytes/well) in the absence or presence of MSCs at a ration of 1:0.1 ($3 \times 10^4$ MSC/well) for 5 and 7 days, with and without 100 µg GM-CSF and IL-3. At specific time points, monocytes were transferred into the FACS tubes; washed twice (1 x PBS at 1200 rpm for 10 minutes), and labelled with 2µl Annexin V-FITC and PI for 15 minutes. The labelled cells were re-suspended in 500µl of the isotonic binding buffer; 104 events were acquired in LSR Fortessa flow cytometer and analysed using the FACS Diva software.

### Statistical analysis

All experiments were conducted with a minimum of three technical repetitions. T-test was used to determine the statistical significance of the data with $p<0.05$.

## RESULTS

### Mesenchymal stem cells inhibit monocyte proliferation in a dose-dependent manner

The proliferation of monocytes at in vitro was induced by GM-CSF and IL-3 alone and in combination. The active monocyte proliferation was observed on day five onwards in the presence of GM-CSF and IL-3. Although, monocytes have responded towards both stimulation individually, yet the optimal monocyte proliferation was noted when costimulated with 100 µg GM-CSF/IL-3 cocktail (data not shown). In the presence of MSCs, monocyte proliferation was significantly inhibited in a dose-dependent manner (Figure 1), while MSC conditioned medium failed to elicit any significant changes in monocyte proliferation. In the highest inhibition of monocyte proliferation, where only 15% of monocyte expansion was observed when the ratio of monocytes: MSCs was 1:0.2. Despite, the inhibition of monocyte proliferation has gradually reduced in higher ratio, yet recorded a significant inhibition in all ratio. Although a notable inhibition was found when MSCs-derived supernatant was used instead of the MSCs, it did not produce a significant change as compared to the positive control.

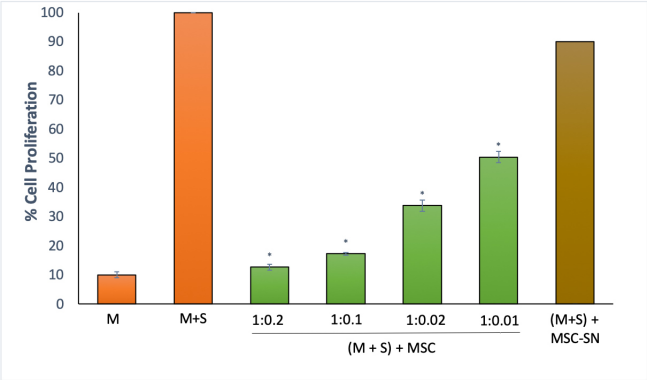


**Figure 1:** Stimulated primary monocyte (M+S) (100µg GM-CSF/IL3 cocktail) served as positive control were cultured in the absence and presence of MSCs. In co-culture, the number of monocyte were fixed (5x104) and MSC were diluted to 5x, 10x, 50x and 100x (1:0.2, 1:0.1, 1:0.02 & 1:0.01). MSCs were plated in a reducing order into 96-well plate overnight and the next day co-cultured with CD14+ cells for seven days. Stimulated monocytes were also cultured in MSC-conditioned medium (MSC-SN). Resting monocytes (M) served as a negative control. This result is representative of three repeated individual experiments with mean ± SD (*$p \leq 0.05$).

### Mesenchymal stem cells arrest monocytes in the $G_0/G_1$ phase of cell cycle

The antiproliferative effect of MSCs on monocyte was further investigated by deciphering the cell cycle status. It was observed that a substantial fraction of stimulated monocytes acquired a high DNA content on day 7 (Table 1) in which the DNA labelling of PI showed 20% of cells in S phase and 10% of cells in the $G_2$/M phase. Whereas in the presence of MSCs, there was a dramatic

**Table I : Mesenchymal stem cells arrest monocytes in the G0/G1 phase of cell cycle**

| Days | Cell Cycle Phases | % of Cells | | | |
|---|---|---|---|---|---|
| | | M | M+MSCs | M+S | M+S+M-SCs |
| DAY 3 | $G_0/G_1$ | 95.0 ± 2.0 | 97.0 ± 1.0 | 96.1 ± 1.0 | 97.0 ± 1.2 |
| | S | 3.2 ± 1.1 | 2.0 ± 0.9 | 1.0 ± 0.3 | 1.2 ± 1.0 |
| | $G_2/M$ | 1.5 ± 0.8 | 0.5 ± 0.7 | 2.0 ± 1.0 | 1.9 ± 0.2 |
| DAY 5 | $G_0/G_1$ | 98.3 ± 1.2 | 97.0 ± 1.0 | 94.0 ± 1.0 | 93.8 ± 0.7 |
| | S | 1.0 ± 0.5 | 1.1 ± 0.4 | 3.5 ± 1.0 | 4.1 ± 0.2 |
| | $G_2/M$ | 0.8 ± 0.3 | 1.3 ± 0.5 | 3.0 ± 0.6 | 2.1 ± 0.8 |
| DAY 7 | $G_0/G_1$ | 96.0 ± 1.0 | 95.0 ± 0.6 | 70.1 ± 3.0 | 89.0 ± 1.0* |
| | S | 3.1 ± 1.0 | 3.0 ± 0.8 | 20.0 ± 0.8 | 8.0 ± 0.5* |
| | $G_2/M$ | 1.0 ± 2.0 | 1.0 ± 3.0 | 10.0 ± 2.5 | 2.4 ± 1.5* |

M-Monocytes, MSCs-Mesenchymal stem cells, S-Stimulation, GM-CSF & IL-3

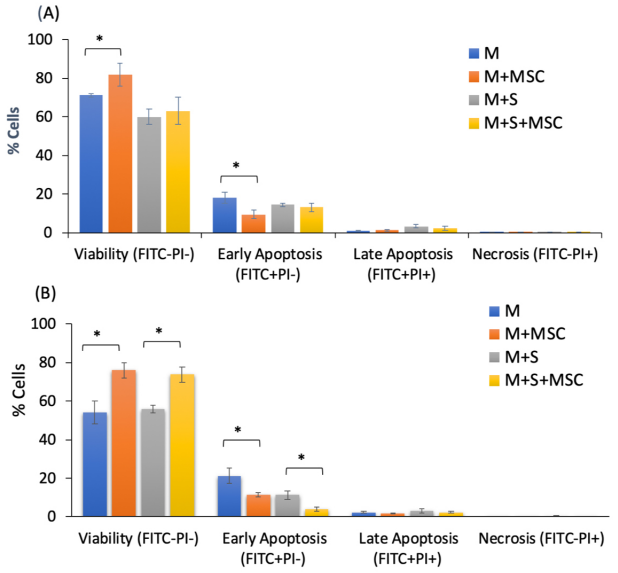


**Figure 2 :** Monocytes ($3x10^5$cells) were stimulated with GM-CSF/IL-3 (100µg/ml), cultured in 6 well plate in the absence and presence of MSC. These cells were incubated for (A) 5 and (B) 7 days and analysed for the apoptosis markers. Results are presented as percentage (%) cells viable (FITC-PI-), undergoing early apoptosis (FITC+PI-), late apoptosis (FITC+PI+) and necrosis (FITC-PI+). At days 7, MSCs significantly (*p ≤ 0.05) enhanced monocyte viability and inhibited early apoptosis. The result is an average of 3 repeated individual experiments with mean ± SD (*p ≤ 0.05).

decrease in the S (8%) and $G^2$/M (2.4%) phases of the cell cycle. In control and test assays of monocyte cultures (resting and stimulated) at days 3 and 5, monocytes were accumulated in the $G_0/G_1$ phase regardless of the presence or absence of MSCs.

**Mesenchymal stem cells protect monocyte from apoptosis**

The percentage of apoptosis in stimulated monocytes culture was evaluated by measuring the expression of early apoptotic markers, phosphatidylserine, along with the integrity of the cell membrane. Phosphatidylserine is a lipid-based molecule found at the inner layer of the cell membrane, upon induction of apoptosis phosphatidylserine exposes to the outer layer of the cell membrane, thus binds to Annexin V. During apoptosis, the integrity of cell membrane compromised, and allow the surpassing of PI molecule that potentially binds with DNA. The combination of Annexin V-FITC and PI staining will further determine the early, and late stages of the apoptosis. Monocytes were stimulated with 100µg GM-CSF/IL-3; in the presence and absence of MSCs at a ration of 1:0.1. At day 5, MSCs significantly enhanced the resting monocyte viability and inhibited their early apoptosis while the stimulated monocyte remained unaffected by MSCs (Figure 2). When the assay progressed to day seven, MSCs significantly enhanced the viability and inhibited the early apoptosis of resting and stimulated monocytes. Cells undergoing late apoptosis and necrosis were negligible and remained unaffected even in the presence of MSC, as shown in Figure 2.

## DISCUSSION

Cellular proliferation has been central to the adaptive immunity, where antigen-activated T and B cells expand into multiple copies of cells in order to deliver adequate effector cell functions. However, the abundant number of innate immune cells such as neutrophils and monocytes does not necessitate the requirement of the cellular expansion in the periphery, since these cells are produced in bone marrow following stimulus from the demands. Hence, human monocytes were generally perceived as non-proliferating cell type (15, 27-29). However, few studies have exposed that there is a small percentage of human monocytes termed as an immature subpopulation of proliferative monocytes. These proliferative monocytes can enter the cell cycle in vitro, in response to M-CSF and GM-CSF (30). These proliferating monocytes could potentially enter the sites of inflammation and contribute to the local macrophage proliferation, which has been observed clinically and in animal models of inflammation (7, 31) (32, 33).

In line with this, the present study also revealed that human monocyte supplemented with GM-CSF and/or IL-3 at various concentrations for seven days undergo a profound cellular expansion. The result from the current study as well as that reported by Clanchy and team, support the existence of a subpopulation of monocytes that corresponded to the external stimuli through cell proliferation (30). We reported that human monocytes, which were isolated via CD14 positive selection proliferate in the presence of external stimuli, GM-CSF/IL-3 upon seven days of culture. However, when human MSCs were added into monocytes co-culture at the beginning, the proliferation of monocyte was inhibited in a dose-dependent manner.

Similarly, we have also conducted the standard proliferation assays in the presence and absence of MSCs at three and five days (data not shown). However, there was no significant proliferation observed when monocytes were activated with external stimuli for 3 and 5 days. Thus, it was unable to deduce the robustness of MSCs in eliciting an antiproliferative function on monocytes as early as possible. It has been demonstrated that MSCs are able to exert a potent antiproliferation on T cells as early as three days of activation, which is entirely reliant on the type of stimulation (34).

Concurrently, the conditioned media harvested from MSCs culture did not inhibit monocyte proliferation significantly. Although it was noted that soluble factors partially contribute to the MSC mediated inhibition, yet the current study highlights the importance of cross-talk via physical contact between MSCs and monocytes. This also confirmed that MSCs mediated immunosuppression is more pronounced with cell-to-cell contact. The present study was unable to pin down a specific signalling pathway which could be exploited by MSCs to deliver such antiproliferative activity. Despite, the antiproliferation activity exerted by MSCs was further deciphered through cell cycle analysis.

It could be possible that cell proliferation driven by GM-CSF/IL-3 in monocytes could have interfered by a common or specific cell cycle mechanism. The present study has exhibited that GM-CSF/IL-3 activation drives the monocytes into cell cycle at day 7 as compared to the resting monocytes. The continuous assessment of cell cycle at day 3, 5 and 7 of activated monocytes indicated that the active cell cycle only takes place on day 7. The results from cell cycle assay have coincided well with the proliferation data, where a significant expansion of monocytes was only detected at day 7 onwards. Likewise, MSCs profoundly arrested the activated monocytes at the $G_0/G_1$ phase of cell cycle and prevented the entry into S and $G_2$/M phases. Both proliferation and cell cycle data indicated that MSCs exhibit a profound antiproliferative activity on primary monocytes, via cell cycle arrest mechanism. A similar result was also observed in B, T and dendritic cells where the cell cycle inhibition appears to take place at the $G_0/G_1$ phases (15, 16). However, it was unable to separate further the $G_0$ and $G_1$ phases of the cell cycle to specifically address the exact phase where the cells are accumulated. The $G_1$ is a transient platform where cells enter into the active cell cycle by equipping with vital proteins such as enzymes. It could be possible that MSCs prevented monocytes from exit the resting phase ($G_0$) to an activation phase ($G_1$) or allow the monocytes to be activated but prevent the DNA synthesis phase (S). However, based on cell cycle data, a significant fraction of the cells remains in the $G_0/G_1$ phases, and only 8.0% of cells progressed to S phase as compared to the 20.0% cells in the positive control. This could lead to the assumption that the inhibition of cell cycle might take place in $G_1$ phase of cell cycle. A similar pattern of the cell cycle inhibition also been reported by previous studies in which cell cycle arrest in $G_0/G_1$ phases was noted. The suppression of the cell proliferation and differentiation of T cells and dendritic cells, respectively, was found to be targeting the $G_1$ phase of the cell cycle (16, 20). Thus, the collective evidence and the current study suggest that the profound antiproliferative activity of MSCs on monocytes or other immune cells could serve as a common denominator of their immunosuppressive action during the regulation of inflammatory responses.

Apoptosis plays an essential role in the daily maintenance and development of the immune system by regulating the number of cells in the human body. Monocytes circulate in the bloodstream for 24-48 hours, and in the absence of an appropriate stimulus, they die spontaneously (35, 36). In the present study, apoptosis assay was conducted to investigate the effects of MSCs on monocyte survival. It was observed that MSCs enhanced monocyte viability and reduced apoptosis. It was previously shown that short-lived primary cells such as neutrophils were rescued by MSCs from apoptosis induced by serum deprivation. In the presence of MSCs, the fraction of viable neutrophils increased, while the dead cell population diminished (17). The reduction of apoptosis in activated monocyte population exerted by MSCs could have resulted from the reduction of cell cycle machinery. Although, proliferation is a crucial phase in immune cells' life span, yet cells that expanded, upon completion of the effector function will be subject to the programmed cell death as to maintain immune homeostasis. In other words, cells that are not proliferating might have a higher chance to survive much longer. The current study has not fully explored the mechanistic nature of the anti-apoptotic activity of MSCs, whether the increased viability is due to modulation of the anti and pro-apoptotic proteins and their cognitive signalling pathways. Furthermore, the current study had only evaluated the effect of MSCs on single donor of monocytes, in which the impact of donor variability is still unknown.

## CONCLUSION

The balance between pro and anti inflammatory responses is a key switch that controls immune homeostasis. Monocytes play an important role in orchestrating the early immune response as monocytes are precursors of macrophages, whose function as antigen presenting cells and mediators of inflammation. However, recruitment of monocytes beyond the physiological demand could be detrimental in several pathological conditions such as atherosclerosis, myocardial infarction, arthritis and multiple sclerosis (31, 37, 38). Being an indispensable cell in the innate immunity and regulation of immune responses, inhibition of monocyte proliferation by MSCs

may represent a new clinical approach for interfering with destructive inflammatory reactions.

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