Human Bone Marrow-derived Mesenchymal Stem Cells Suppress T Cell Proliferation by Inducing Cell Cycle Arrest

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ABSTRACT

Objective: Mesenchymal stem cells (MSC) are common residents of bone marrow and are defined by their higher self-renewal ability and multilineage differentiation. MSC play an important role in supporting haematopoiesis and therefore are implicated in influencing immune responses. In line with this, MSC have been utilised to treat graft-versus-host disease (GVHD) in order to suppress unwanted T cell proliferation. In this study, we investigated the immuno-suppressive effect of bone marrow derived MSC on T cell proliferation at the cell cycle level. **Methods**: MSC were generated from human bone marrow and confirmed by their immuno-phenotyping. Resting or PHA stimulated allogeneic peripheral blood mononuclear cells (PBMC) were co-cultured in the presence or absence of MSC. T cell proliferation was accessed by trypan-blue exclusion assay at day three. Consequently cell cycle analysis was carried out to determine the mechanism of anti-proliferation. **Results:** MSC failed to elicit proliferation at resting T cell. However, proliferation of PHA-stimulated T cells was dramatically inhibited in the presence of MSC in a dose dependent manner ($p < 0.05$). Following the inhibitory activity, MSC prevented activated T cells from entering the S phase of cell cycle by arresting them in the G_1 phase. **Conclusions:** Our findings indicate that MSC escape recognition by T cells and inhibit T cell proliferation by cell cycle arrest at G_1 phase. This immuno-suppressive effect is most probably mediated by cell-to-cell contact and/or secreted soluble factors.

Keywords: Mesenchymal stem cells, T cells, immuno-suppression, cell cycle arrest

INTRODUCTION

Mesenchymal stem cells (MSC) are non-haemopoietic stem cells that reside in bone marrow compartment and constitute 0.0001 -0.01 % of total cell population. Traditionally, MSC are characterised by their higher self-renewal ability and differentiation capability into tissues of mesenchymal origin (osteocytes, chondrocytes, and adipocytes).[1] Physiologically, MSC constitute a haemopoietic niche in the bone marrow micro-environment and regulate the survival, self-renewal, migration, and differentiation of haemopoietic stem cells (HSC).[2] Besides, MSC have also been shown to regulate early T cell lymphopoiesis in the bone marrow [3] as well as T cell development in thymus.[4]

The idea of investigating the effect of MSC on T cell responses came from the notion that MSC contributed to thymic epithelial cells which are essential in T cell positive selection.[5,

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[6] In fact, a preferential migration of infused donor MSC into recipient thymus after bone marrow transplantation (BMT) has been observed.[6] Initial studies addressing the immunological properties of MSC showed that they not only fail to stimulate allogeneic T cells but also exhibit an active immuno-suppressive effect. MSC have been tested for their immuno-suppressive activity on T cell responses triggered by allo-antigen, [7-9] peptide antigens,[10] mitogens [7, 11] and CD3/CD28 antibody.[10, 11]

Recently, MSC have been exploited as agents for cellular therapy due to their immuno-suppression properties. MSC have been implicated in clinical trials (especially in stem cell transplantation) as an agent for prevention and treatment of graft versus host disease (GVHD). The clinical trial results are promising as co-transplantation of HLA-identical MSC and haematopoietic stem cells (HSC) had strikingly reduced the incidence of GVHD in haematological malignancy patients.[12,13] Although MSC have been shown to successfully cure GVHD, the mechanism of immuno-suppression in the recipient is still unclear. However, studies from murine model reveal that, MSC-secreted molecules such as hepatocyte growth factor (HGF) and keratonocyte growth factor promote the tissue repairing mechanism and inhibit proliferation of activated lymphocytes in GHVD induced mice.[14, 15] The data from animal work and clinical trials clearly indicate that MSC ameliorate GVHD by inducing anti-inflammatory cascade via inhibitory cytokines and cell-to-cell mediated immune cell anti-proliferation.[14, 16, 17]

Although other studies have shown that the immuno-suppressive activity of MSC directly targets T cell proliferation, the detailed mechanism and the impact on T cell proliferation has yet to be explored. In this study, we evaluated the effects of MSC on T cell proliferation at the cell cycle level.

MATERIAL AND METHODS

Generation of Mesenchymal Stem Cells

Bone marrow samples were purchased from Stem Cell Technologies, USA whereby the samples were obtained in accordance with local ethics requirement. Purchased bone marrow aspirates were diluted with 1X PBS (phosphate-buffered saline) and layered onto Ficoll-Paque PLUS (1.077 g/ml, Amersham Biosciences, Sweden) and centrifuged at 2000 rpm, 10°C for 20 minutes. Mononuclear cells (MNC) were collected, counted, and seeded in 25 cm² vented culture flask (Nunc Brand Products) in MSC complete medium which consisted of Dulbecco's modified Eagle's medium (DMEM) with high glucose, GLUTAMAX-I (Gibco, Invitrogen, USA), supplemented with 10% pre-selected foetal bovine serum (Stem Cell Technologies Inc., London, UK), 1% of penicillin and streptomycin (Gibco, Invitrogen), 0.5% fungizone (Gibco, Invitrogen), and 0.1% gentamycin (Gibco, Invitrogen). After incubation at 37°C in a humidified 5% CO_2 atmosphere for 72 hours, non-adherent cells were removed by replacing the media. When the adherent cells achieved 80 to 90% confluence, cells were trypsinised by using 0.05% trypsin-EDTA (Invitrogen, Canada) at 37°C for 5 minutes, and further expanded in 75 cm² flasks. Homogenous and neat cell population was obtained after 3 to 5 passages. During the expansion, the media was changed every 4-5

days. Expanded cells were used in experiments or cryo-preserved in freezing media (10 % DMSO, 40 % DMEM, 50% foetal bovine serum).

Purification of Human Peripheral Blood Mononuclear Cells

Human peripheral blood samples of 10-20 ml amount were obtained from normal healthy donors. All samples were acquired with written, informed consent in accordance with the UPM, Faculty of Medicine and Health Sciences Ethics Committee requirements. Human peripheral blood mononuclear cells (PBMC) were purified from peripheral blood samples by Ficoll-Paque density gradient separation (1.077 g/ml, Amersham Biosciences, Sweden). PBMC were cultured in complete T cell medium consisting of RPMI 1640 (Gibco BRL,) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin (Gibco BRL), 0.5% Fungizone (Gibco, Invitrogen), and 0.1% Getamicin(Gibco, Invitrogen) or cryo-preserved and thawed prior to experiments.

Immuno-phenotyping

The immuno-phenotyping of the MSC was performed by using the FACSCalibur flow cytometer (Becton Dickinson). MSC were stained with the following monoclonal antibodies: CD105-PE (R&D System), anti-CD73-PE, anti-CD45-PE, anti-CD34-FITC, anti-HLA-ABC-PE-Cy5, anti-HLA-DR,DP,DQ- FITC, anti-CD80-PE, and anti-CD86-APC (Becton Dickinson, Biosciences Pharmingen) for 30 minutes at 2-8°C and washed with 1X PBS. The fluorochrome analysis was included with appropriate PE-, FITC-, PE-CY5-, APC-, conjugated isotype controls. Cell cycle analysis was performed by evaluated intracellular proteins and DNA content by fluorescein isothiocynate (FITC) and propidium iodide (PI) dyes, respectively.[18]

Polyclonal Stimulation of PBMC

In mitogen-induced proliferation assays, responder PBMC (5 x 10^4 cells) were cultured with phytohemagglutinin (PHA) at 5-10 ìg/ml (Sigma Aldrich, USA).

T Cell Proliferation Assay

T cell proliferation assay was performed on 96 well plates (Cellstar). MSC were seeded accordingly to appropriate wells (5 x10^4 cells, 1 x10^4 cells, 1x10^3 cells, and 5x10^2 cells) and incubated overnight to allow adherence to plastic surface. Human PBMC were seeded at fixed numbers into all wells (each well 5 x 10^4 cells) and stimulated with 5μg/ml PHA. A similar assay was also set by replacing MSC with MSC-conditioned medium. Cells were incubated at 37°C in 5 % CO_2 for 3 days and harvested on day 4 for viable cell count by using a haemocytometer and trypan blue solution at 0.4 % (Sigma Aldrich, USA).

Statistical Analysis

The data is expressed as mean ± SEM except where otherwise stated. The unpaired sample student T-test was performed to compare the two mean values. Probability of null hypothesis less than 5 % (p< .05) was considered statistically significant.

RESULTS

Immuno-phenotyping of MSC

The immuno-phenotyping of MSC was determined on early passages of MSC cultures by flow-cytometry. The results are as reported in *Fig. 1*. More than 95% of MSC were positive for CD105 (SH2), CD73 (SH3 and SH4) and negative for the haematopoietic markers CD34 and CD45. MSC expressed major histocompatibility complex (MHC) class I antigens but neither MHC class II antigens, nor co-stimulatory molecules CD80 and CD86.
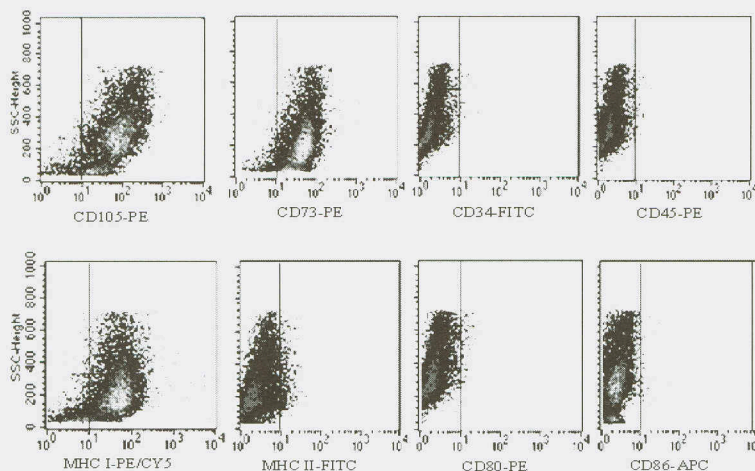

Figure 1. Cell surface markers expression of MSC. MSC expressed CD105, CD73, and HLA class I, but lack expression of CD45, CD34, HLA class II and co-stimulatory molecules (CD80 and CD86).

MSC Failed to Stimulate Resting T Cell Proliferation

The effect of allogeneic MSC on resting T cell was evaluated by adding MSC as third party to PBMC. T cell proliferation induced by allogeneic MSC was measured by cell count after third day of assay. The cell count results indicate that allogeneic MSC at various ratios failed to induce T cell proliferation significantly (*Fig. 2*).

MSC Inhibit Mitogen Stimulated PBMC

T cell proliferation was stimulated by PHA and measured by performing viability cell count on day 4 of culture. As shown in *Fig. 3*, the proliferation of mitogen-stimulated PBMC was suppressed by MSC in a dose dependent manner. In addition, MSC conditioned medium also exhibited anti-proliferative property, although to a lesser extent.
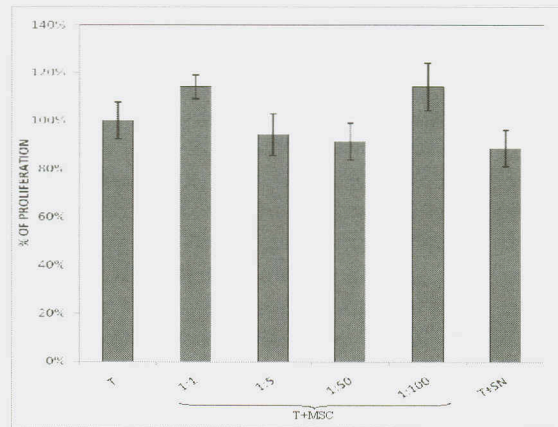
Figure 2. MSC alone failed to stimulate resting T cells proliferation. Fifty thousand of PBMC were co-cultured with MSC for 3 days at different ratios. T cell proliferation was assessed by performing viability cell count at day 4. MSC failed to induce T cells proliferation at resting state of T cells
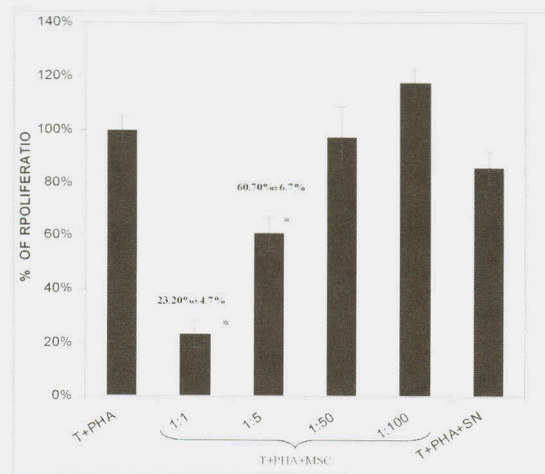
Figure 3. MSC inhibit PHA-stimulated T cell proliferation in a dose-dependent manner. PHA-stimulated T cells were co-cultured with MSC at various ratios (1:1, 1:5, 1:50 and 1:100) for three days. At day 4, the number of T cells was counted by viability cell counting. MSC were replaced by MSC conditioned-medium in order to evaluate immuno-suppressive effect MSC derived supernatant.

MSC Arrest T Cell in G0/G1 Phase of Cell Cycle by Blocking the DNA Synthesis

The selective anti-proliferative effect of MSC on T cells prompted us to dissect this effect at cell cycle level. The cell cycle was assessed by intracellular protein and DNA staining using FITC and PI, respectively. PBMC (2x106) were stimulated by PHA in the presence or absence of MSC (0.1x106). After 72 hours of incubation, the T cells were harvested for cell cycle analysis. In the absence of MSC, a large proportion of T cells became blasts and acquired high protein and DNA content (*Fig. 4*) which indicated active cell cycle progression. This was confirmed by PI staining which showed 50.48 % of cells in S phase and 17.69 % of the cells at G2/M phase (*Fig. 5*). In the presence of MSC, protein synthesis was not affected; however, there was a dramatic decrease in DNA synthesis. Only 35.81 % cells were positive for PI staining whereas in the absence of MSC about 50.48 % cells were PI positive. Consistent with the reduced DNA content, MSC also prevented the T cells entering S phase (35.81 %) and further decreased G2/M phase (14.74 %).

DISCUSSION

Over the past two decades, MSC have been extensively investigated for their potential use in multiple organ regeneration and to a lesser extent, as a cellular therapy for congenital disorders. Additional importance has also been given to MSC as they can suppress an immune response and have been exploited in treating graft versus host disease (GVHD) after the allogeneic transplantation.[13] Animal studies of cerebral injury, myocardial infarction, muscular dystrophy and bone fractures appear to confirm their potential clinical usage.[19] Furthermore, phase I clinical study with MSC demonstrate that they are non-toxic and non-immunogenic to recipients.[20] In line with phase I study, administration of autologous and third party MSC into osteogenesis imperfecta patients corrected bone disorder thus resulting in a successful phase II clinical trial.[21] More interest has been attracted to MSC profound immuno-suppression effects activities upon a broad range of immune cells, including T cells,[7, 8, 10, 18, 22-26] B cells, [27] natural killer (NK) cells, [28, 29] and dendritic cells.[30]

Our data demonstrates that MSC escape immune recognition by T cells, thus failing to elicit significant proliferation of resting T cells (*Fig. 2*). This phenomenon may be due to the fact that, MSC lack expression of co-stimulatory molecules, CD80 and CD86 (*Fig. 1*), which is crucial for T cells activation. Nevertheless, the addition of anti-CD 28 mAb as a exogenous co-stimulatory or transduction with CD80 and CD86, did not enhance the ability of MSC as antigen presenting cells.[8, 25]. However, T cell proliferation stimulated by mitogen was actively inhibited by MSC implying that MSC is less immunogenic. The inhibitory efficiency of MSC was in proportion to the dose of MSC whereby MSC inhibited T cell proliferation in a dose-dependent manner. Previous studies also show similar data. [7, 8]

Interestingly, our data also showed that MSC conditioned-medium or supernatant collected from MSC cultures slightly reduced the proliferation of activated T cells. This finding indicates that the immuno-suppressive effect of MSC on T cells is mediated by soluble factor(s). However, we could not disregard the fact that MSC may partially require cell cell-to-cell contact since the magnitude of inhibition is mounted to the same degree when MSC are physically in contact with T cells. In line with our results, others have
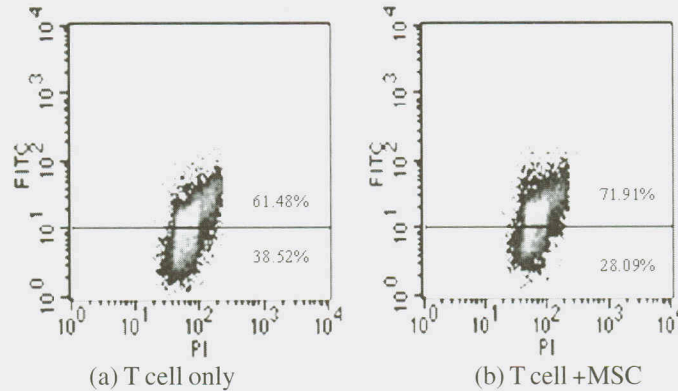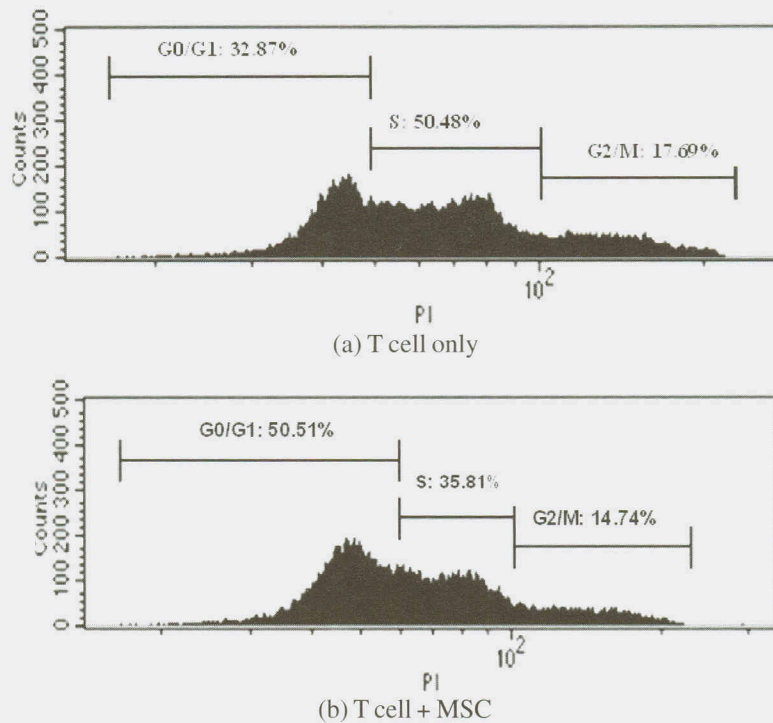
(a) T cell only   (b) T cell +MSC

Figure 4

(a) T cell only

(b) T cell + MSC

Figure 5

Figures 4 & 5. Effects of MSC on the cell cycle. T cells (2×10^6 cells) were stimulated with 5µg/ml PHA in the absence and presence of MSC (1×10^5 cells). After 72 hours, T cells were harvested, fixed, and stained with FITC and PI for protein and DNA content quantification. Intracellular protein and DNA content of T cell were assessed by flow cytometry

shown that MSC mediate the immuno-suppression by secreting soluble factors.[7, 8] However, the possible soluble factor(s) that may be involved in inhibitory mechanism remain to be elucidated. Several candidates have been proposed and tested for their role in mediating immuno-modulation such as transforming growth factor (TGF)-β1[7] and hepatocyte growth factor (HGF),[7] prostaglandin (PGE$_2$),[16] and indolamine-deoxyegenase (IDO).[24]

We have also demonstrated that the immuno-suppressive effect of MSC on T cells is more likely directed at the proliferation level of T cells. The effects of MSC on T cell division/cell cycle were assessed by FITC and PI staining which measures the intracellular protein and DNA content of T cell respectively. The cell cycle analysis showed that MSC did not alter the protein synthesis of PHA-stimulated T cell but blocked the DNA synthesis. Thus, T cells were arrested in G$_1$ phase of the cell cycle and were unable to progress throughout the S phase (*Fig. 5*). These data is further supported by Glennie's study whereby MSC induce T cell anergy by arresting T cell in G$_1$ phase in amurine model.[18] In addition, MSC have also shown to inhibit B cell proliferation by arresting them in the G$_0$/G$_1$ phase of the cell cycle [27] and block monocytes from entering G$_1$ phase and arresting them in G$_0$ phase.[30] Although MSC exerted immuno-suppressive effects on different immune cells and this occurs at different phases of the cell cycle, yet, overall findings support the notion that MSC do influence the cell cycle status of immune cells. Furthermore, this similar mechanism of anti-proliferative has been observed in tumour cells as well. [30, 31]

In conclusion, our data suggest that MSC escaped immune recognition and suppressed T cell proliferation triggered by polyclonal stimuli. This inhibitory effect was mainly directed at cell cycle level of T cells and arrested the T cells in the G$_1$ phase. Although, this effect could be mediated by both physical contact and soluble factor(s), yet the underlying mechanisms remain to be elucidated.

ACKNOWLEDGMENTS

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