Mesenchymal Stem Cells Do Not Suppress Lymphoblastic Leukemic Cell Line Proliferation

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ABSTRACT

Background: Several studies have demonstrated the immunosuppresive effects of mesenchymal stem cells (MSCs) in allogeneic or mitogenic interactions. Cell-cell contact inhibition and secretion of suppressive soluble factors have been suggested in this regard. **Objective:** To investigate if adipose derived MSCs could inhibit Jurkat lymphoblastic leukemia T cell proliferation during coculture. **Methods:** Adherent cells with the ability of cellular growth were isolated from normal adipose tissues. Initial characterization of growing cells by flow cytometry suggested their mesenchymal stem cell characteristics. Cells were maintained in culture and used during third to fifth culture passages. Jurkat or allogeneic peripheral blood mononuclear cells (PBMCs) were labeled with carboxy fluorescein diacetate succinimidyl ester and cocultured with increasing doses of MSCs or MSC culture supernatant. Proliferation of PBMCs or Jurkat cells under these conditions was assessed by flow cytometry after 2 and 3 days of coculture, respectively. **Results:** Results showed the expression of CD105, CD166 and CD44, and the absence of CD45, CD34 and CD14 on the surface of MSC like cells. Moreover, initial differentiation studies showed the potential of cell differentiation into hepatocytes. Comparison of Jurkat cell proliferation in the presence and absence of MSCs showed no significant difference, with 70% of cells displaying signs of at least one cell division. Similarly, the highest concentration of MSC culture supernatant (50% vol/vol) had no significant effect on Jurkat cell proliferation ($p>0.6$). The same MSC lots significantly suppressed the allogeneic PHA activated PBMCs under similar culture conditions. **Conclusion:** Using Jurkat cells as a model of leukemia T cells, our results indicated an uncertainty about the suppressive effect of MSCs and their inhibitory metabolites on tumor or leukemia cell proliferation. Additional systematic studies with MSCs of different sources are needed to fully characterize the immunological properties of MSCs before planning clinical applications.

Keywords: Mesenchymal Stem Cells, Jurkat Cells, Suppression

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INTRODUCTION

Over 40 years ago, Friendenstein and colleagues showed that transplanted bone marrow contains a population of heterogeneous cells with properties of self-maintenance and self-renewal, called mesenchymal stem cells (MSCs) (1). In humans, MSCs are derived from different organs and tissues; however, at the moment bone marrow and adipose tissues are the main sites of MSC isolation. In bone marrow, MSCs represent 0.001%-0.01% of the total cell population (2,3). They are plastic-adherent, spindle-shaped cells that express a series of key markers used for their characterization in different studies, although there is no unique immunophenotype marker (2,4).

Mesenchymal stem cells have high growth plasticity and are able to differentiate into lineages of other tissues (3-9). Many potential applications have been suggested for various clinical situations, such as tissue regeneration and hematopoietic stem cell transplantation (10,11). Adherent bone marrow-derived stromal cell cultures supported the production of hematopoietic progenitor cells over a period of several weeks to months (8). In this connection, the simultaneous injection of donor marrow stromal cells with hematopoietic stem cells accelerates the recovery of hematopoiesis after total body irradiation (12,13).

A major breakthrough in MSC application was the finding that they are able to suppress immune cells (7). Mesenchymal stem cells are able to arrest T cell division induced by alloantigens or mitogens (14-16), inhibit the differentiation and maturation of dendritic cells (17), reduce the proliferation and immunoglobulin expression of B cells (18), inhibit the formation of cytotoxic T cells by secreting soluble factors, but are not able to interfere with CTLs and NK cell lysis (19). They also decrease the production of inflammatory cytokines by various immune cell populations (20). The exact mechanisms underlying such effects of MSCs have not been clearly identified; however, many studies concur that cell-cell contact-dependant mechanisms and the secretion of soluble factors are involved in MSC suppression (7,8,21). Among the soluble factors released by MSCs that may affect cell proliferation are hepatocyte growth factor, indole amine dioxygenase, and inhibitory cytokines such as transforming growth factor-β and interleukin-10 (22-26).

Because MSCs are able to downmodulate the immune system, it is possible to exploit these cells for the treatment of autoimmune diseases (7,10). Moreover, the homing property of MSCs toward tumor sites raises the possibility of using MSCs for tumor therapy and tumor suppression. However, the inhibitory action of MSCs on the immune system may interfere with this application and enhance tumor growth by suppression of immune cells (27). More studies are needed to predict the inhibitory effects of MSCs under different conditions. Therefore, we designed the present study to investigate whether normal human adipose-derived MSCs could inhibit or stimulate the proliferation of Jurkat leukemia T cells when cocultured with them.

MATERIALS AND METHODS

Isolation and Culture of Human Adipose-Derived MSCs. Informed consent was obtained from patients scheduled for abdominoplasty to use part of their discarded tissue for research purposes. Fragments of adipose tissue were washed with phosphate-

buffered saline (PBS), minced into small pieces, and digested with 0.2% collagenase (Gibco, UK) at 37 °C on a shaker for 2 h. The resulting suspension was centrifuged at 400 × *g* for 10 min. Stromal cells were purified by Ficoll gradient centrifugation at 400 × *g* for 30 min and cultured in DMEM medium (GIBCO, UK) containing 10% fetal bovine serum (GIBCO) and 100 IU/mL penicillin and 100 μg/mL streptomycin (Biosera, UK). Nonadherent cells were discarded after 24 h of culture. The adherent cells were cultured by changing the medium every 4 days and harvesting the cells in passage 3 after almost 30 days of culture. All MSCs used in this study were under their fifth culture passage. The culture supernatants (SN) from MSC cultures were derived from 7-day cultures of different lines and kept frozen at −70°C until use in Jurkat cultures.

Jurkat Cell Culture and Maintenance. The Jurkat leukemia T cell line (28) was cultured in RPMI 1640 medium (Biosera, UK) supplemented with fetal bovine serum, penicillin and streptomycin as above. Jurkat cell cultures were kept at 37 °C in a humidified incubator with 5% CO_2. At approximately 80% confluence, cultured cells were harvested and used to seed fresh cultures.

Labeling Jurkat Cells and PBMCs with Carboxy Fluorescein Diacetate Succinimidyl Ester (CFSE) Dye. Washed Jurkat cells or PBMCs were resuspended at 2 $\times 10^7$ cells/mL in PBS and mixed 1:1 with CFSE at 5 μM. Cell suspensions were incubated for 15 min at 37 °C with frequent agitation. An equal volume of complete RPMI culture medium was added to the cells and left for 1 min at room temperature to stop the labeling process. The cells were washed 3 times and resuspended in complete culture medium.

Coculture of MSCs with Jurkats or Allogeneic PBMCs. Mesenchymal stem cells from 3 independent donors' adipose tissues at densities of 1.5×10^5 and 3×10^5 were plated in 24-well plates 24 h prior to coculture with Jurkat cells. Carboxy fluorescein diacetate succinimidyl ester-labeled Jurkat cells were added to MSC preloaded wells at a concentration of 1.5×10^6 cells per well in complete culture medium. In this way, the ratios of MSC to Jurkat cells were estimated at 1:5 and 1:10, as suggested by others (29).

To assess the inhibitory effect of metabolites in MSC culture supernatant (SN), serial concentrations (10%, 30% and 50%) of culture SNs were added to separate Jurkat cultures in a total volume of 1.5 mL. Jurkat cell proliferation was studied after 2 and 3 days of culture by flow cytometry.

Similarly, to show the suppressive effect of MSCs, allogeneic peripheral blood mononuclear cells (PBMCs) were separated by Ficoll gradient, labeled with CFSE and cocultured with MSCs in the presence of PHA as a stimulator. PHA activated PBMC cultures without MSCs were considered as controls. Proliferation of PBMCs was analyzed by flow cytometry on day 3 of culture.

Immunophenotyping of MSCs and Flow Cytometry Studies. To observe the expression of different markers by MSCs, cells were harvested by mild trypsinization (Biosera, UK). The expression of CD14, CD34, CD44, CD45, CD105 and CD166 was analyzed by immune staining of cells with appropriate fluorescent conjugated monoclonal antibodies (all supplied by BD Biosciences, USA) and flow cytometry. The same cells also were stained with fluorochrome-labeled isotype-matched antibodies to exclude nonspecific staining as a negative control. Moreover, cultured MSCs were able to differentiate to hepatocytes under the influence of specific differentiation and growth factors (data not shown).

CFSE labeled PBMCs or Jurkat T cells were harvested from cocultures with MSCs at different time points, washed twice with PBS and analyzed by flow cytometry.
Flow cytometry was done with a 3-color BD FACS Calibur machine using Cell Quest software (Becton Dickinson, USA) for data acquisition, and WinMDI v. 2.8 software for data analysis and presentation.
Statistical Analysis. The statistical analysis was performed by paired *t*-test using SPSS software (v. 11.5). The data are presented as the mean ± SE. A *P*-value <0.05 was considered statistically significant.

RESULTS

Phenotype of Adipose-Derived MSCs. We used flow cytometric studies to show the presence or the absence of a panel of different cell markers to partially identify the cells purified from adipose tissues. Established cells were shown to express CD105, CD166 and CD44 whereas they showed no significant expression of CD45 lymphocyte marker, CD34 heamatopoietic stem cell and endothelial marker and CD14 monocyte-macrophage marker (Figure 1). Moreover, these cells were able to differentiate to hepatocytes in preliminary differentiation studies. These results together could suggest the growing cells to be MSCs.

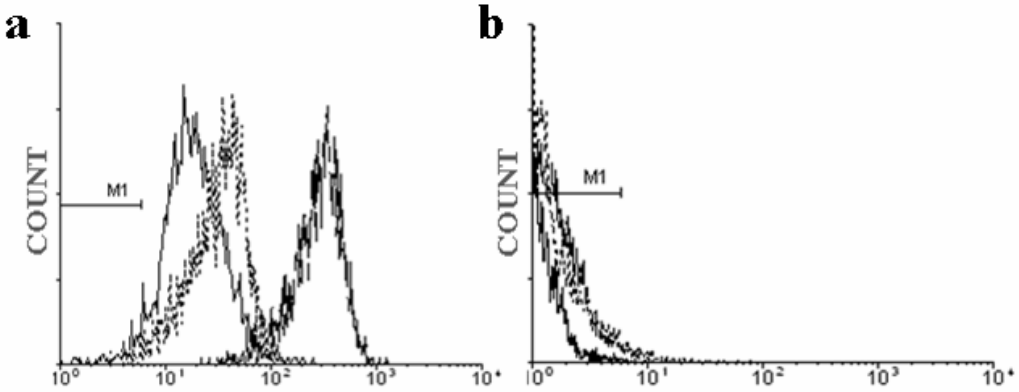

Figure 1. A typical example of flow cytometric characterization of cultured mesenchymal stem cells. Mesenchymall stem cells were purified from adipose tissue samples of abdominoplasty operations and cultured as explained in Materials and Methods. Expression of surface markers was analyzed by flow cytometry.
a: Cells were shown to express various amounts of: CD44 (solid bold), CD105 (solid narrow) and CD166 (dotted); b: Cultured MSCs were negative for CD14 (solid bold line), CD34 (solid narrow line) and CD45 (dotted line) expression. M1: Isotype control marker.

Unlike PBMCs, Jurkat Cell Proliferation can not be Inhibited by MSCs. Mesenchymal stromal cells were plated in duplicates 24-h prior to coculture with the CFSE-labeled Jurkat T cell line at MSC/Jurkat ratios of 1:5 and 1:10. Jurkat cells were harvested on day 2 and day 3 post-culture to assess their proliferation by flow cytometry (Figure 2). Figure 3 shows the mean percentages of dividing Jurkat cells in the presence of MSCs in 3 independent experiments, compared to control Jurkat cell cultures. The results at both time points were similar on day 2 post-culture, 77% of Jurkat cells showed at least one cell division. The presence of MSCs did not significantly suppress

Jurkat cell proliferation, and up to 70% of the cells showed signs of at least one cell division at both time points ($p>0.3$) (Figure 3). A slight decrease in the cell proliferation was observed in cultures at a MSC/Jurkat ratio of 1:5. Presence of the same lots of MSCs in coculture with allogeneic PHA activated PBMCs could suppress the cell proliferation up to 75% on days 3 post culture. Figure 4a and 4b demonstrate typical examples of PBMC growth and its suppression by MSCs from multiple experiments. Figure 4c summarizes similar results from those experiments.

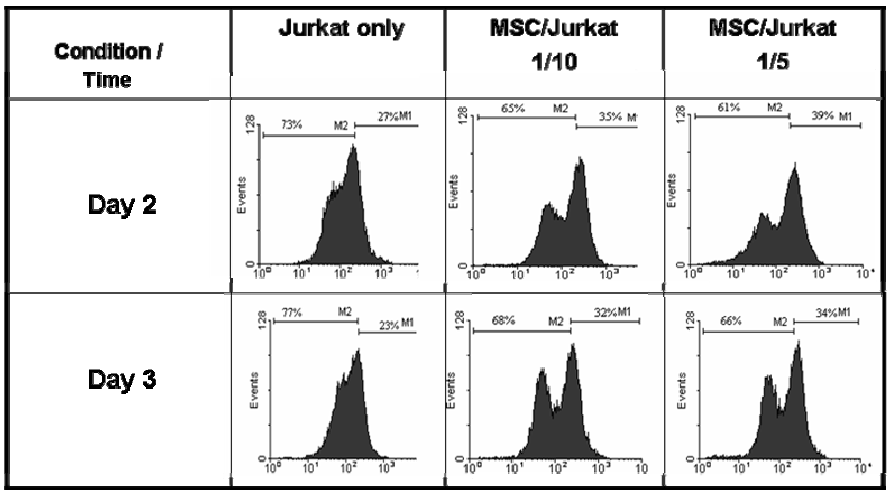

Figure 2. A typical example of CFSE analysis for Jurkat proliferation in cocultures of Jurkat cells and MSCs.
To achieve MSC/Jurkat ratios of 1:5 and 1:10, 3×10^5 and 1.5×10^5 MSCs, respectively, were plated in 24-well plates 24 h prior to coculture with 1.5×10^6 CFSE-labeled Jurkat T cells. Cells were harvested on days 2 and 3 post-culture and their proliferation was measured by flow cytometry. M1 and M2 show the percentages of nondivided and dividing populations, respectively.

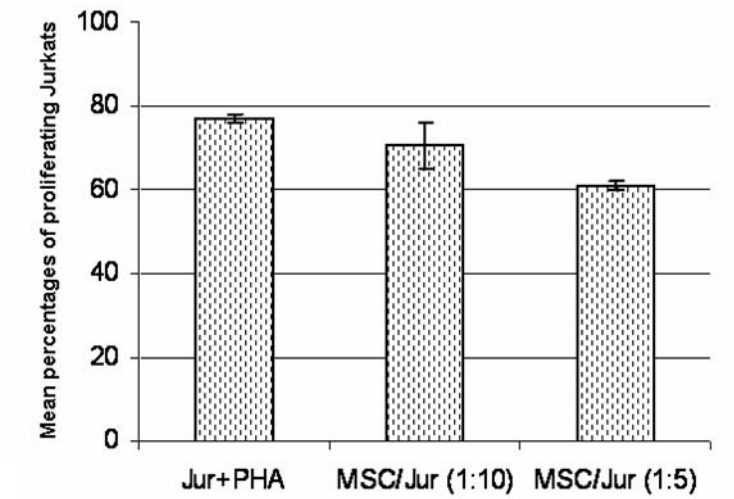

Figure 3. Effect of MSCs on Jurkat proliferation.
Bars show mean percentages of proliferating Jurkats in coculture with MSCs. PHA activated Jurkats (first column from left) were used as control culture. Results are from 3 independent experiments using 3 different MSCs. The presence of MSCs did not significantly change Jurkat cell proliferation ($p>0.05$).

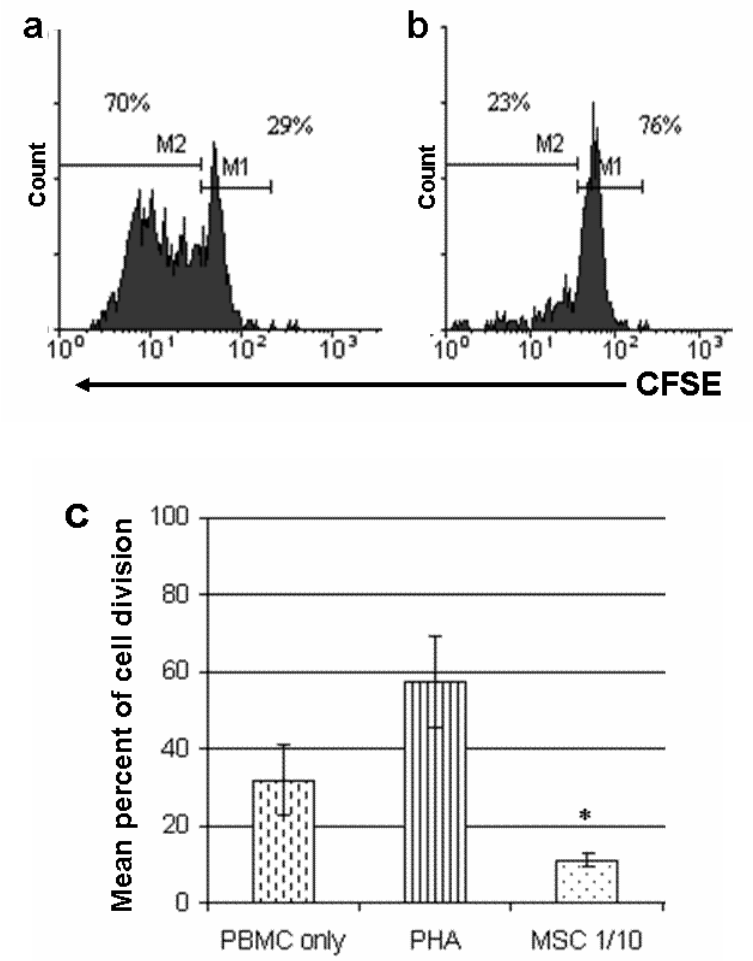

Figure 4. Typical example of CFSE analysis for hPBMCs proliferation suppression by cultured MSC. Human PBMCs were labeled with CFSE prior to coculture with MSCs. PBMCs were activated by PHA and harvested at day 3. Proliferation of harvested CFSE-labeled PBMCs was assessed using flow cytometric analysis. In the absence of MSCs (a) majority of cells showed significant cell proliferation while in the presence of MSCs (b), they caused massive prevention. Multiple independent experiments (n = 6) with different MSCs produced similar results (c).

MSC Culture Supernatant has no effect on Jurkat Cell Proliferation. To assess the possible inhibitory effect of MSC culture SN on Jurkat cell growth, cultures with different concentrations of SN from 4 different MSCs were prepared in quadruplicates. Figure 5 shows a typical example of how Jurkat cells were growing in the presence of different concentrations of MSC culture SN. Similarly, Figure 6 shows the mean percentages of 4 independent experiments of Jurkat cell proliferation with 10%, 30% and 50% of MSC culture supernatants (vol/vol of the total culture medium). Jurkat cell proliferation was analyzed by flow cytometry on days 2 and 3 post-culture. As shown, even the highest concentration of SN (50% vol/vol) did not significantly affect Jurkat cell proliferation ($p>0.6$), and more than 70% of the cells showed at least one cell division. However, the same culture supernatants from MSCs were able to significantly suppress the growth of allogeneic PBMCs (up to 55% of the original growth without MSCs or the MSC SN) after PHA activation.

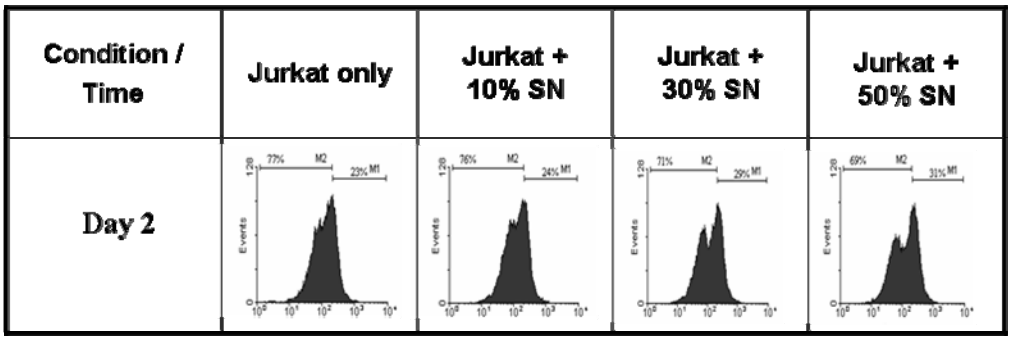

Figure 5. A typical example of CFSE analysis for Jurkat cell proliferation in the presence of MSC culture supernatant on day 2 of coculture.
Different concentrations of MSC supernatant were added to cultures of 1.5 $\times 10^6$ CFSE-labeled Jurkat T cells. Jurkat cells were harvested on days 2 and 3 post-culture and their proliferation was measured by flow cytometry. Results from two time points were similar.

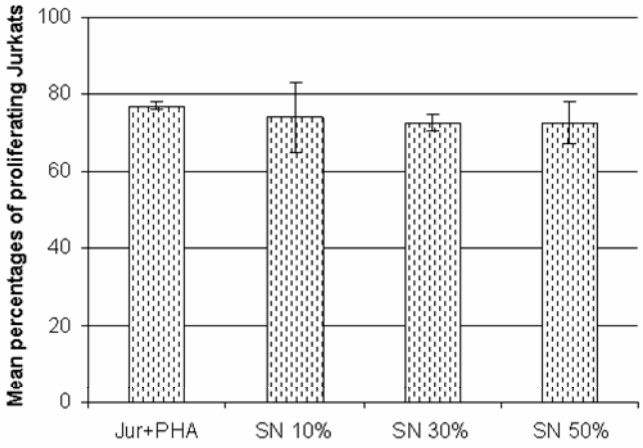

Figure 6. Effect of MSC culture supernatant on Jurkat cell proliferation.
Bars show mean percentages of proliferating Jurkats in coculture with MSCs. PHA activated Jurkats (first column from left) were used as a control culture. Results are from 4 independent experiments. The presence of MSC SN did not significantly change Jurkat cell proliferation ($p<0.05$ was considered significant).

DISCUSSION

Mesenchymal stem cells are heterogenous cells present in different tissues which can differentiate to produce diverse arrays of mesenchymal and nonmesenchymal cell lineages. Several lines of immunological research have shown that these cells can arrest lymphocyte cell proliferation. The exact mechanism of this effect has not been elucidated. In this study we used the Jurkat T leukemia cell line as a model to analyze and compare the inhibitory effect of MSCs, as previously reported by other investigators (30). We demonstrated that Jurkat T cell growth could not be suppressed by the immunomodulatory effects of MSCs or their culture SN. In fact, none of 3 independently established MSC lines prepared from different donors could inhibit Jurkat T cell growth during 3 days of culture, nor could MSC culture SN. Benvenuto et al. (30) found that bone marrow-derived MSCs were able to inhibit Jurkat cell proliferation. In their experiments, MSCs apparently inhibited anti-Fas monoclonal antibody-mediated apoptosis and

thus decreased the proportion of annexin-V-positive cells. However, fat-derived MSCs such as the cell types we used in the present study may not be an entirely comparable model, so other mechanisms should be sought to explain the inability of our MSCs to inhibit Jurkat cell growth. We only partially characterized the MSCs and this may raise doubt about the nature of these cells and their biological function, although multiple flow cytometric analyses using monoclonal antibodies against specific markers of MSCs or lineage-specific markers showed the cells to be potentially MSCs. Moreover, as a test of the immune inhibitory effect of cultured MSCs in parallel experiments, we found our MSCs fully competent in suppressing the growth of normal allogeneic peripheral blood mononuclear cells when stimulated with phytohemagglutinin. This means our MSCs retained their immune inhibitory effect in the culture; therefore the reason for their inability to inhibit Jurkat cell proliferation must lie elsewhere. Jurkat T cells are immortalized leukemia cells with fast proliferative capacity, and their rapid cell cycle may abrogate the suppressive mechanisms of the MSCs. Moreover, this alteration may have induced mechanisms to evade immunotolerant signaling imposed by MSCs. In addition to that, the original Jurkat cell line used in this study could have a mixture of different cell types with different sensitivities toward MSC inhibition. Even with an effective suppression of some cells, others may overgrow and repopulate the culture. Complementary studies including the cloning of this cell line and similar cell lines are needed to better answer this controversy and clarify the exact mechanisms of this event.
Having different biological features, such MSCs hold great promise for broad application in different disorders, although the exact mechanism of their activities such as cell proliferation suppression remains controversial even among different species (31). Showing the inability of MSCs on Jurkat cell proliferation in this study, can not contradict the previous observations about this effect on lymphocytes and other cell types. However, it may warn us about the necessity of further characterization of these cells before their widespread use for human trials.

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