Immunomodulatory Effects of Mice Mesenchymal Stem Cells on Maturation and Activation of Dendritic Cells

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) possess a wide range of immunomodulatory functions mostly in immune cells including dendritic cells (DCs). DCs are the key cells in the immune response and play an important role in initiating cell-mediated immunity. **Objective:** To evaluate the immunomodulatory effects of MSCs supernatant on maturation and function of DCs. **Methods:** Bone marrow derived mice MSCs were isolated and cultured. Twenty-four, forty-eight and seventy-two hours after passage 6, supernatants were collected and MSCs were assessed by cytometric analysis for the expression of CD34, CD44, CD45 and SCA-1. Splenic DCs were isolated using MACS and then co-cultured with MSCs supernatant. Expression of CD86, CD40 and MHC-II on DCs were also evaluated by cytometry. H^3-thymidine incorporation by proliferating T cells was determined in two separate MLR assay settings. In one setting, DCs were co-cultured with T cells in the presence of MSCs supernatant, and in the other setting DCs were treated with MSCs supernatant and then were co-cultured with T cells. Production of IL-12, IL-6 and IL-10 cytokines was measured in the supernatant of DCs treated with MSCs supernatant. We also measured IFN-γ and IL-4 levels in MLR supernatant. **Results:** The results showed that 72h MSCs supernatant could decrease the expression of MHC-II and CD86. The T cell proliferation was inhibited in the presence of MSCs supernatant and MSCs supernatant treated DCs as demonstrated by MLR assay. A significant increase in IL-4 level and a non significant decrease in IFN-γ level in MLR supernatant were observed. However, IL-6, IL-10 and IL-12 production did not change significantly. **Conclusion:** MSCs supernatant has a time dependent effect on the maturation of DCs. Also, it could alter cytokine production from responding T cells toward Th2. Generally, the findings of this study supported the immunomodulatory effect of MSCs supernatant on DCs maturation and function.

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INTRODUCTION

Bone marrow derived Mesenchymal Stem Cells (MSCs) are pluripotent stem cells that are released to in the blood circulation and are implanted into damaged tissues in response to the damage associated molecular patterns (DAMP). By the expression of some proteins such as IL-10, IDO and PGE-2, MSCs have the ability to decrease the inflammation and to repair damaged tissue (1,2). MSCs have unique characteristics such as immunoregulatory properties and low immunogenicity (1-4). Recently, MSCs were brought into attention in transplant surgery because of their immunomodulatory abilities against T cells (3), B cells (1), DCs (1,4) and natural killer (NK) cells (4). These cells are currently being used to reduce immunological transplant rejection and to prolong graft survival (5). DCs are known as main antigen presenting cells (APCs) having an important role in induction of the adaptive immune response. According to the expression of co-stimulatory molecules and cytokine production, they are divided into three groups namely immature (iDCs), semi-mature (smDCs) and mature DCs (mDCs). Mature DCs express a high level of co-stimulatory molecules and are able to induce T cell proliferation. In contrast, immature and semi-mature DCs express lower co-stimulatory molecules and induce tolerance upon interaction with T cells (6,7). Notably, DCs cultured in the presence of MSCs, display a surface phenotype that revealed a poor degree of differentiation, maturation and function (8-10). In agreement with previous data, the surface density of the maturation markers CD83, CD80 and CD86 were significantly lower in cells cultured in the presence of MSCs (1,8,11). DCs with tolerogenic properties (immature and semi-mature DCs) are intended for maintenance of grafts and prevention of autoimmune diseases (12). It is shown that MSCs culture supernatant decreases the expression of B7 co-stimulatory molecules and MHC-II by DCs and induces tolerogenic DCs (Tol-DC) to produce high levels of IL-10 (13,14). Some studies have demonstrated that MSCs can exert their immunoregulatory effects on maturation and function of DCs and T cell proliferation by soluble factors such as IL-6 and M-CSF (15,16). Indeed, IL-6 is involved in the reversion of the maturation of DCs to a less mature phenotype and in partial inhibition of the conversion of bone marrow progenitors into DCs (16). Previously, several studies have shown that the expression of IL-10 is significantly increased when MSCs and T cells are co-cultured (17,14). Thus, MSCs may inhibit DCs maturation through the action of the IL-10 (17). *In vivo* administration of MSCs results in the inhibition of pathogenic antigen-specific T cells, as shown in a mouse model allograft rejection (18), in the experimental autoimmune encephalomyelitis (19), in graft-versus-host disease (20) and in collagen-induced arthritis (21). However, little is known about the *in vivo* mechanisms involved. Although, a number of recent studies have focused on the effect of MSCs on DCs maturation and function (8,11,22,23), data has been confounded by differences in models, technical approaches and particularly by the isolation protocols of MSCs or DCs. In this study, we evaluated if secretory proteins present in mice bone marrow derived MSCs culture supernatant could inhibit the maturation of splenic mice DCs and decrease allo-reactive responses in the MLR assay.

MATERIALS AND METHODS

Mice. Five- to six-week-old BALB/c and C57BL/6 mice were purchased from Razi Institute (Shiraz, Iran). All mice were inbred and maintained under specific pathogen free and standard conditions. All of the experimental procedures on handling the animals were approved by the animal ethical committee of Shiraz University of Medical Sciences.

Isolation and Culture of Bone Marrow Derived Mesenchymal Stem Cells. Bone marrow cells were isolated from femurs and tibias of BALB/c mice. Isolated cells were cultured in 25 cm^2 flasks in low glucose Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), sodium pyruvate (1%), glutamine (1%) and penicillin-streptomycin (100 μg/ml) in a CO2 incubator at 37°C. After 48 hours, non-adherent cells were removed. Adherent cells were grown to 80-90% confluency, then trypsinized and passaged (24). In passage 6, the purity of the cells was checked by Sca-1, CD44, CD45 PE labeled and CD34 FITC labeled antibodies. The supernatant of MSCs in passage 6, was collected after 24, 48 and 72 h of trypsinization and kept at -70°C.

In Vitro Multilineage Differentiation Studies. For adipogenesis assay, BM-MSCs were cultured in DMEM supplemented with 1 μM dexamethasone, 0.5 μM ascorbic phosphate, 200 μM indomethacin and 10% FCS for 14 days. Osteogenesis was evaluated in the appropriate induction media including 1 μM dexamethasone, 10 mM β-glycerolphosphate, 0.5 μM ascorbic phosphate and 10% FCS in DMEM for 14 days. The differentiation phenotype was documented using oil red O staining for adipocytes, and alizarin for osteocytes.

Splenic DCs Isolation. Gradient media, Nycodenz (Axis Shields, Norway) and MACS were used to isolate the splenic DCs. We cultured DCs in accordance with our protocol previously described in detail elsewhere (25). Mice spleens were chopped and digested with 1 mg/ml collagenase D (Roche, Germany) and 2 μg/ml DNase (Roche, Germany), then meshed with 0.2 μm sieve. Cells were washed with RPMI 1640 (Sigma, St. Louis, MO, USA) containing 5 mM EDTA. Cell pellet was resuspended in RPMI 1640 with 10% fetal calf serum (FCS) and 5 mM EDTA. The cell suspension was layered on Nycodenz and centrifuged at 1800 rpm and 4°C for 20 min. The interface layer was collected and purified by anti-CD11c micromagnetic beads (Miltenyi Biotec, Germany) (25). The purity of isolated DCs was checked by analyzing the expression of CD11c molecule using lowcytometry (BD Biosciences, USA).

Flowcytometry Analysis. Isolated DCs were incubated overnight with either 24, 48 or 72 h MSCs supernatants with or without LPS (250 ng/ml), in 6-well plates and then harvested and stained with Phycoerythrin (PE)-conjugated anti-CD11c, fluorescent isothiocyanate (FITC)-conjugated anti-CD40, FITC-conjugated anti-CD86 and FITC-conjugated anti-I-A/I-E (mouse MHC II) and appropriate conjugated isotypes, all from BD Pharminogen (San Diego, CA). Data were analyzed using Win Midi software (Scripps, La Jolla, CA). Forward- and side-scatter parameters were used to gate live cells. The mean fluorescence intensity (MFI) for different markers was compared with that of a negative control (25). Data were analyzed using FlowJo software. Percent and mean fluorescent intensity (MFI) for different markers were compared with negative control (DC). Reported percentages and MFIs are representative of the ratio of percent or MFI of the test /Percent or MFI of the corresponding control groups.

Mixed Lymphocyte Reaction (MLR). T lymphocytes were isolated from lymph nodes of C57BL/6 mice using nylon wool. The purity of cells checked by staining with FITC-conjugated anti-CD3 antibody (BD Pharminogen, USA) was more than 90%. Mitomycin (0.5 mg/ml) was added to MSCs supernatant-treated DCs for 20 min, then cells were washed with phosphate buffered saline (PBS) and resuspended in the culture medium containing 15% fetal calf serum to use as stimulating cells. MLR assay was performed by plating these DC cells in triplicate at a concentration of 10^4 cells/well in a 96-well round bottomed culture plate (Nunc, Denmark co-cultured with 10^5 allogeneic T cells for 48 h. MLR tests were done under two different conditions: In the first condition, isolated DCs were treated overnight with either of the 24 h, 48 h or 72 h MSCs supernatants. Then they were co-cultured with T cells for 48 h. In the second condition, untreated DCs were co-cultured with T cells in the presence of either 24 h, 48 h or 72 h MSCs supernatants. Negative controls were a series of triplicate wells containing allogeneic T cells and DCs untreated with MSCs supernatants. T cell proliferation was measured by H^{3-}thymidine proliferation assay (26).

Cytokine assays. The supernatant of supernatant treated DCs, with and without LPS and supernatant of MLR assay, were collected and used to measure cytokines using an enzyme linked immuosorbent assay (ELISA) according to the manufacturer's protocol. IFN-γ (eBioscience, USA) and IL-4 (eBioscience, USA) levels were measured in the supernatant of MLR assay. IL-12 (eBioscience, USA), IL-10 (eBioscience, USA) and IL-6 (eBioscience, USA) levels were also estimated in the supernatant of MSC- treated DCs, with and without LPS. The sensitivities of IL-4, IFN-γ, IL-10, IL-6 and IL-12 kits were 4, 15, 30, 4 and 15 pg/ml, respectively.

Statistical Analysis. All data were representatives of three independent experiments presented as mean ± standard deviation (SD). The differences between groups were analyzed by Mann-Withney and Kruskal-Wallis tests using Graph Pad Prism 5 software (Graph-Pad Software Inc, San Diego, CA). P values less than 0.05 were considered as significant.

RESULTS

Immunophenotypic Characterization of BM-MSCs. Cells isolated from bone marrow were analyzed for the expression of cell surface antigens reported as determinants of MSCs phenotypic profile. These cells displayed strong expression of typical MSC markers like Sca-1 and CD44 (Figure 1 A and B) while they were negative for the hematopoietic markers such as CD45 and CD34 (Figure 1 C and D). The MSCs purity was more than 95% in passage 5 (Figure 1).

Potential for Osteogenic/Adipogenic Differentiation. Adipogenic differentiation of BM-derived MSCs was demonstrated by the formation of triglyceride-containing vacuoles in the cell cytoplasm, visualized by Oil Red O staining (Figure 2A). In contrast, the control cells maintained only in culture medium and without adipo-inductive factors, did not display an adipogenic phenotype. Osteogenic differentiation of BM-derived MSCs was induced by treatment with osteo-inductive factors.

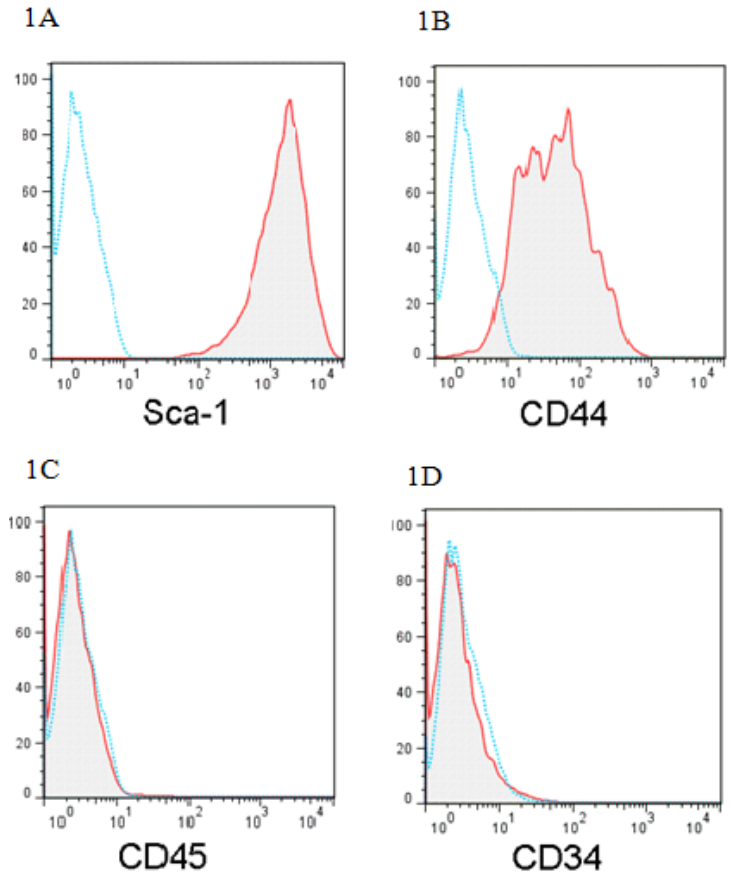

Figure 1. Purity of MSCs was checked using anti Sca-1, CD44, and CD45 PE labeled antibodies and anti CD34 FITC labeled antibody (A-D). The result showed almost 95% of cells in passage 5 were positive for Sca-1 and CD44 and negative for CD45 and CD34.

The osteogenic phenotype was demonstrated by calcium accumulation detected by Alizarin-red S staining (Figure 2B). BM-derived MSCs cultured in medium without specific inducing factors were negative after this staining. MSCs are pluripotent cells with fibroblast-like shape in culture medium (Figure 2C).

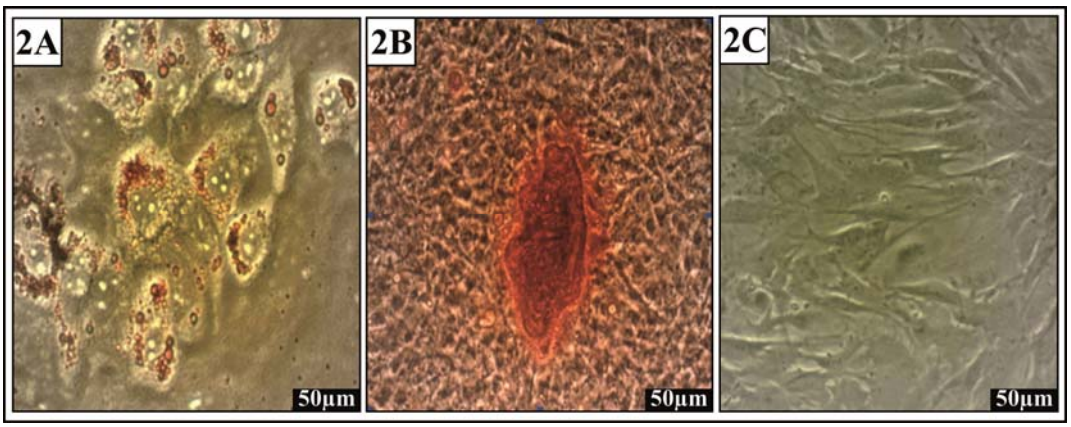

Figure 2: Differentiation potential of MSCs obtained from the bone marrow. Adipogenic differentiation of MSC was shown by Oil red O staining of adipocytes (2A, ×400). Osteogenic differentiation of MSC was revealed by Alizarin red S (2B, ×400). Un-differentiated mice MSCs in passage 6 are shown in part 2C (×400).

Effects of MSCs culture supernatant on the expression of co-stimulatory molecules on DCs. More than 90% of splenic cells after DCs isolation were CD11c positive. The results showed no significant changes on the expression percentage and mean fluorescence intensity (MFI) (data not shown) of MHC-II molecules observed in treated DCs compared to the control group (DC only, Figure 3). However, the expression of MHC-II molecules on DCs treated with 48 h MSCs supernatant plus LPS decreased in comparison to 24h MSCs supernatant and LPS treated DCs (p=0.05). The MHC-II expression was decreased significantly (p=0.05) in 72 h MSCs supernatant treated DCs without LPS compared to 48 h MSCs supernatant treated DCs (Figure 3).

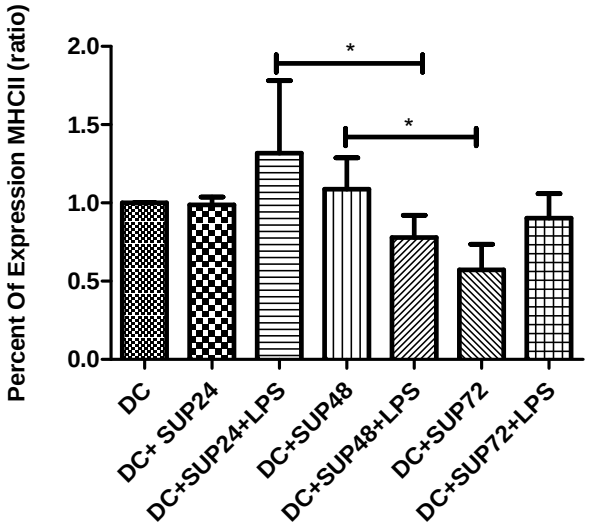

Figure 3. DCs isolated from mice spleen were treated with 24 h, 48 h and 72h MSCs supernatant with or without LPS. The data showed MHC-II expression percentage was decreased in DCs treated with 72h MSCs supernatant in comparison with 48h MSCs supernatant, without LPS (p=0.05). As shown, the expression percentage of MHC-II was decreased in condition treated with 48h MSCs supernatant in comparison with 24h MSCs supernatant in presence of LPS (p=0.05). The results are representative mean ± SD of three independent tests. (Supernatant = Sup)

DCs treated with 72 h MSCs supernatant and LPS expressed lower level of CD86 in comparison with DCs treated with 24 h MSCs supernatant and LPS (p=0.05, Figure 4). The expression of CD86 was decreased in conditions treated with 24h and 48h MSCs supernatant with LPS in comparison with 24h and 48h MSCs supernatant without LPS, respectively (both p=0.05, Figure 4). The percentage and MFI of CD40 molecules expression on DCs were not affected by MSCs supernatant treatment (data not shown).

Effect of MSCs Supernatant on T Cell Proliferation in MLR Assay. The purity of isolated T cells was more than 90%. DCs function can be characterized in part by their ability to stimulate alloreactive T cells in MLR assay. To determine whether MSCs supernatant affected the DCs allostimulatory activity, MLR assay was performed. In the present study, we evaluated the optimum ratios of DC/T at 1:40, 1:20, 1:10, 1:5 and 1:2.5 ratios and observed that 1:10 ratio was the best. To evaluate the function of DCs, MLR tests were done under two different conditions:

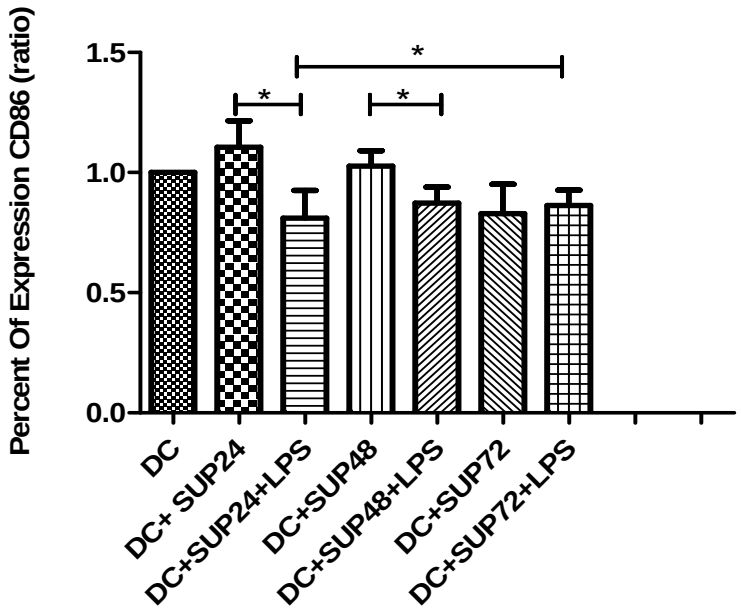

Figure 4. DCs isolated from mice spleen were treated with 24, 48 and 72 h MSCs supernatant with or without LPS. The data showed CD86 expression ratio was decreased in DCs treated with 72h MSCs supernatant compared to 24h MSCs treated condition with LPS (p=0.05). As shown, the expression of CD86 was decreased in conditions treated with 24h and 48h MSCs supernatant with LPS in comparison with 24 h and 48 h MSCs supernatant without LPS, respectively (both p=0.05). The result are representative mean ± SD of three independent tests. (Supernatant = Sup)

First, we cultured DCs with T cells and then the supernatant of MSCs was added to them and finally T cell proliferation was assessed. In the second condition, MSCs supernatant was added to DCs overnight and then MLR test was performed and then T cell proliferation was evaluated.

Under conditions where T cells were co-cultured with untreated DCs in the presence of MSCs supernatant, the allogeneic T-cell stimulatory index was significantly increased in the presence of 24 h MSCs supernatant compared to the control (238.1 ± 51.89 vs. 100 ± 5.39, $p<0.05$). Furthermore, the allogeneic T cell stimulatory index was significantly decreased in the presence of 72 h MSCs supernatant compared to the 24 h MSCs supernatant (76.79 ± 38.20; vs. 238.1 ± 51.89; $p<0.05$, Figure 5A). Under conditions where T cells were co-cultured with treated DCs, the allogeneic T-cell stimulatory index was significantly increased in the presence of DCs treated with 24 h and 48 h MSCs supernatants compared to the control (205.0 ± 21.2% and 200.4 ± 19.5 %, respectively, $p<0.05$). Interestingly, the allogeneic T-cell stimulatory index was significantly decreased in the presence of the 72 h MSCs supernatant compared to the control and the 24 h and the 48 h MSCs supernatant (22.0 ± 5. 5%) ($p<0.05$, Figure 5B).

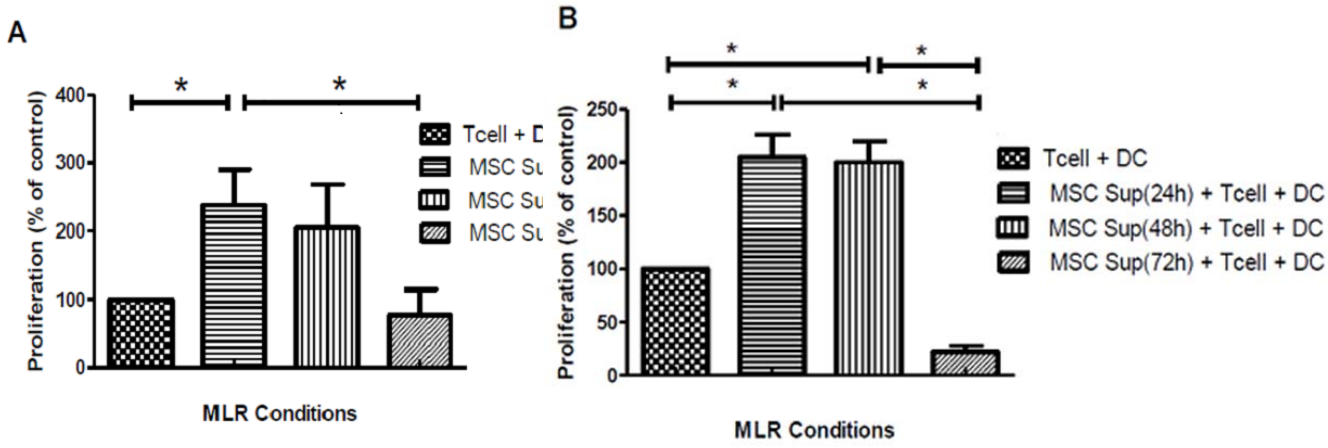

Figure 5. The results showed proliferation (% of control) of T cells in co-culture with DCs and MSCs supernatant. A: T cell proliferation decreased when treated with 72h MSCs supernatant in comparison with 24h MSCs supernatant ($p<0.05$). As shown in this figure, T cell proliferation increased in the presence of 24h MSCs supernatant in comparison with control (Untreated condition) ($p<0.05$). B: T cell proliferation in co-culture with 72h MSCs supernatant treated DCs diminished in comparison with 24h and 48h MSCs supernatant treated DCs ($p<0.05$). Presence of MSCs supernatant for 24 and 48 h, enhanced T cell proliferation comparing the control group (Treated condition) ($p<0.05$). The data are representative mean ± SD of three independent tests (Supernatant = Sup)

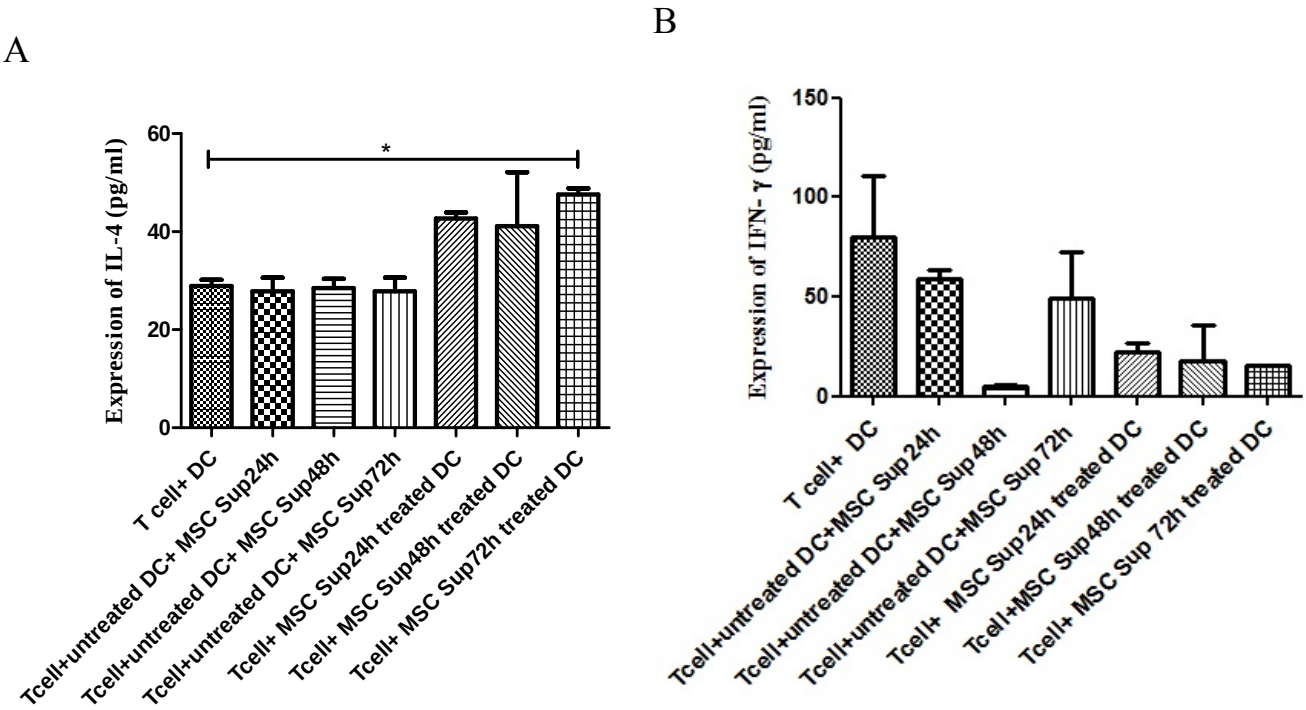

Figure 6 (A-B). Levels of IL-4 and IFN-γ in MLR condition. (A): IL-4 was increased in condition with T cells which co-cultured with 72h MSCs supernatant treated DCs compared to control ($p=0.05$). (B): IFN-γ was decreased in MLR assay condition treated with 48h MSCs supernatant in comparison with control (T cell with untreated DC), but this change were not significant. The data representative mean ± SD of three independent tests. (Supernatant: Sup).

Cytokine Assays. Because DCs are responsible for the polarization of T cells toward Th1 or Th2, we assessed if allostimulation of the DCs treated with MSCs supernatants could alter cytokine production from the responding T cells. The secretion of IL-4 was significantly increased in MLR assay by T cells co-cultured with the 72 h MSCs supernatant treated DCs compared to the control (47.58 ± 1.257 vs. 27.81 ± 2.828, p=0.05). The level of this cytokine was increased by T cells co-cultured with the 24 h and the 48 h MSCs supernatant treated DCs compared to the control but differences were not statistically significant (Figure 6A).

In MLR assay, the level of IFN-γ decreased in T cells co-cultured with DCs in the presence of the 48 h MSCs supernatant compared to the control (47.58 ± 1.257 vs. 27.81 ± 2.828, Figure 6B). Assay of cytokines in the supernatant of DCs treated with MSCs supernatants showed no significant changes in IL-12, IL-10 and IL-6 production either in the presence of LPS or in its absence (Figure 7A-C).

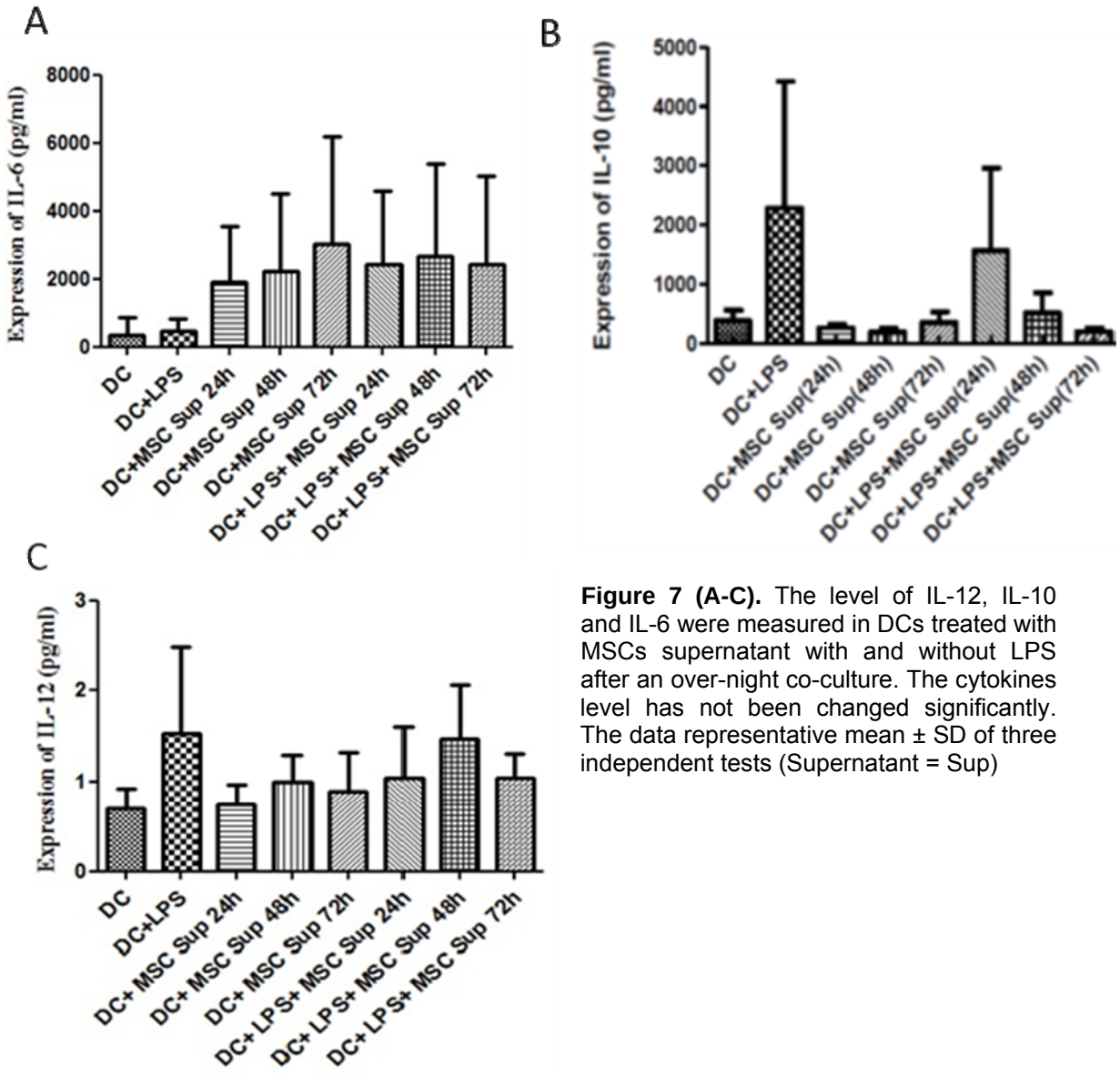

Figure 7 (A-C). The level of IL-12, IL-10 and IL-6 were measured in DCs treated with MSCs supernatant with and without LPS after an over-night co-culture. The cytokines level has not been changed significantly. The data representative mean ± SD of three independent tests (Supernatant = Sup)

DISCUSSION

DCs are the links between innate and adaptive immune responses. T cell mediated immunity is strongly related to the maturation of DCs. Immature DCs cannot initiate T cell mediated immunity and even induce tolerance in T cells. Having therapeutic effects in DC therapy (7,27,28). Evidence shows that MSCs suppress immune responses, but the underlying immunomodulatory mechanisms are not fully understood. Some studies have indicated that soluble factors are essential for enhancing the suppressive effect of human MSCs, while the effect of rodent MSCs is mediated by cell-cell contact (3,24). Most of the inhibitory soluble factors are not constitutively secreted by MSCs, but they can be induced by the interaction between activated effector cells and MSCs.

Dinicola *et al*. investigated the immunomodulatory effect of MSCs and showed that MSCs suppressed T cells proliferation and also inhibited monocyte-drived DC generation (29). MSCs induce generation of Tol DCs and down-regulate the expression of MHC II, CD80, CD86 and CD40 but the mechanism of their action has not been well defined (4,13). In the present study, we evaluated the immunomodulatory effect of soluble factors of mouse MSCs culture supernatant on immature DCs and examined the progression of DCs maturation. The results showed that the 72 h culture supernatant of MSCs decreased the expression of MHC-II and CD86 on DCs. The expression of CD40 and the MFI of these molecules were not affected by the culture supernatant of MSCs (Data not shown). Also, T cell proliferation was inhibited by DCs treated with the 72 h MSCs culture supernatant compared to the control and the 48 h and the 24 h MSCs culture supernatants. Because DCs are responsible for polarization of T cells toward Th1 or Th2 lineages, we assessed if the allostimulation of the DCs treated with the culture supernatant of MSCs could alter cytokine production by the responding T cells. In an MLR assay, we showed that secretion of IL-4 was increased by T cells co-cultured with DCs which were treated with 24, 48 and 72 h MSCs supernatant as compared to the control group, but the differences were only statistically significant in the presence of the 72 h MSCs supernatant. Interestingly, secretion of IL-4 was increased in DCs treated with 72 h MSCs supernatant, as well. We also found a non-significant decrease in the secretion of IFN-γ by T cells co-cultured with DCs in the presence of the 48 h MSCs supernatant compared to the control. Previous studies reported that IL-6 (together with M-CSF), produced by MSCs, would play a major role in the MSC-mediated inhibition of DC differentiation (8,16). MSCs are reported to constitutively produce a significant amount of IL-6 and this was highly increased when MSCs were co-cultured with monocytes. IL-6 is involved in the reversion of the maturation of DCs to a less mature phenotype and in the partial inhibition of the differentiation of bone marrow progenitors to DCs (16). The interfering effect of neutralizing IL-6 and M-CSF specific mAbs on the MSC-mediated inhibition of DCs differentiation has also been investigated (30). Some studies have also suggested that MSCs specifically inhibit DCs function and maturation through IL-10 and the JAK-STAT signaling pathway (1). The expression of CD80, CD86, MHC-II and CD11b/c declined in the presence of IL-10. DCs generated in the presence of MSCs express low levels of IL-12, TNF-α and MHC-II and high levels of anti-inflammatory IL-1β and IL-10 (31). Thus, IL-10 can antagonize IL-12 during induction of inflammatory immune response (32). In our study, however, the assay of cytokines in the supernatant of DCs treated with MSCs supernatant showed no significant changes in IL-12, IL-10 and IL-6 production.

In conclusion, the aim of this study was to investigate the immunomodulatory effects of MSCs secretory factors on DCs. Our results showed time dependent immunomodulatory effects of MSCs supernatant. We showed that secretion of IL-4 was increased and IFN-γ decreased, but the expression of IL-12, IL-10 and IL-6 did not change in the presence of the 72 h culture supernatant of MSCs. Since the MSCs supernatant was shown to affect phenotypical and functional properties of DCs and T cells in this research, it can be concluded that the MSCs supernatant has immunomodulatory effects on these cells. It should be noted that immunotherapy with MSCs supernatant is still ambiguous and requires further studies. However, because of the various side effects of MSC therapy, it appears that its supernatant may be safer to use.

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