

TGF- β 1 Transduced Mesenchymal Stem Cells Have Profound Modulatory Effects on DCs and T Cells

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) and transforming growth factor- β 1 (TGF- β 1) molecules are well known for their immunomodulatory properties and their function in tissue regeneration and remodeling. **Objectives:** To evaluate the interaction of TGF- β 1 engineered MSCs with T cells and dendritic cells (DCs) and their modulatory effect on the immune response. **Methods:** MSCs and DCs were generated from bone marrow of Balb/c mice and T cells were generated from mice lymph nodes. TGF- β 1 expressing lentiviruses were used for MSCs transduction, and then these engineered MSCs were co-cultured with T cells and DCs. T cells proliferation and cytokines release and also DCs maturation, TNF- α release, and stimulation of allogeneic T cells were evaluated. **Results:** T cells proliferation and IFN- γ release were suppressed by TGF- β 1/MSCs while IL-4 secretion was enhanced. Co-cultured DCs with TGF- β 1/MSCs showed reduced expression of CD40, CD86, and MHC II and also lower level of TNF- α secretion. Co-cultured DCs could also induce lower levels of allogeneic T cells proliferation and IFN- γ release in comparison to control DCs. **Conclusion:** Engineered TGF- β 1/MSC cells showed collaborative immune suppressive functions between TGF- β 1 and MSCs to modulate T cells and DCs immune responses. We therefore suggest that TGF- β 1/MSC cells could provide a promising tool for treatment of clinical conditions such as organ transplantation, GVHD, and autoimmune disorders.

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Keywords: Dendritic Cells, Immunomodulation, Mesenchymal Stem Cells, Transforming Growth Factor- β 1, T Cells

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INTRODUCTION

Mesenchymal stem cells (MSCs) are a heterogeneous population of multi-potent cells which have self-renewal capacity and differentiation potential into several mesenchymal lineages including bones, cartilages, adipose tissues and tendons (1). MSCs could repair tissue injuries and also prevent immune cell activation and proliferation. The suppressive actions of MSCs are exerted through cell-cell contact and soluble factors (e.g., IL-6, prostaglandin E2, nitric oxide) (2,3). Immunomodulatory properties of MSCs have led to realizing the true potential of MSC-based cell therapy. The use of MSCs as immunomodulators has been explored in cell/organ transplant, tissue repair, autoimmune diseases, and prevention of graft vs. host disease (GVHD) (1,4). Basically, MSCs exert modulatory effects on both innate and adaptive immune cells and responses. Some of MSC effects include: inhibition of CD4⁺ T cell proliferation in response to mitogens (5), inhibition of IL-2, TNF- α production by T cells (6), induction of classic CD4⁺CD25^{hi} FOXP3⁺ T regulatory cells (T-regs) differentiation (7), inhibition of B cell function and differentiation (8), and inhibition of dendritic cells (DCs) generation from monocytes and co-stimulatory molecules expression (9). On the other hands, one of the main mediators of tissue remodeling and immunosuppression functions is transforming growth factor- β 1 (TGF- β 1). TGF- β 1 is synthesized and secreted by a wide variety of cells including immune cells, and its importance in immune regulation and tolerance has been increasingly recognized. TGF- β 1 has multiple suppressive effects on T cells, B cells, macrophages, and increased TGF- β 1 production correlates with protection and/or recovery from autoimmune diseases. TGF- β 1 acts to terminate immune responses and the passage from effector to memory T cells. It down-regulates adhesion molecules and inhibits adhesion of leukocytes to endothelial cells (10,11). With regard to these backgrounds, in the present study we engineered MSCs with TGF- β 1 gene and then evaluated engineered MSCs properties in regulating T cells and DCs function.

MATERIALS AND METHODS

Animal. Six to eight week old inbred Balb/c mice were purchased from Pasteur Institute (Tehran, Iran) and kept in our animal laboratory at 23°C and 12-hour light/dark cycle. All of the methods used in this study, were approved by Animal Care and Use Committee at Tarbiat Modares University.

Production of TGF- β 1 Engineered Lentiviruses. Mouse TGF- β 1 (GenBank: BC013738.1) cDNA inserted into pCR4-Topo plasmid vector was purchased (ImaGene, USA). Using SmaI and BamHI restriction enzymes TGF- β 1 gene was successfully sub-cloned into the p240 (pLOX-EWgfp modified vector) lentivirus transfer vector (Addgene, USA) and then the insertion was confirmed by electrophoresis and sequencing. Applying plox-MD2 (Addgene, USA) plox-PAX2 (Addgene, USA) packaging vectors, and p240-TGF- β 1 vectors, recombinant lentiviruses (LVs) were produced in HEK293T cell line. The supernatant of infected HEK293T cells was then concentrated by ultracentrifugation. Expression of GFP (i.e. green fluorescent cells aka GFP⁺) was assessed to monitor the transduced cells by immunofluorescence microscopy as well as titrating LVs by flowcytometry.

MSC Generation and Transduction. MSCs production from bone marrow of BALB/c mice (n=5) was done by sequential culture method (12). In brief, bone marrow cells were flushed from the medullary cavities of femurs and tibias and cultured in DMEM (DMEM-LG, Invitrogen, USA) supplemented with 11 mM sodium bicarbonate, 2 mM l-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum for 3 h in a 37°C and 5% CO₂ incubator. Then, nonadherent cells were removed carefully and fresh medium was replaced. A purified population of MSCs obtained 3 weeks after the initiation of culture. The cells were negative for CD31, CD34, CD11b, CD45, and CD135 antigens and positive for CD44 and Thy1.2 (CD90). In addition, the potential of isolated cells to differentiate into osteogenic and adipogenic lineages was examined. 1.5×10^6 purified MSCs/ml (from passage 2-3) was cultured for 24 h in a 6-well plate containing DMEM prior to transduction by concentrated LVs. After that MSCs were transduced in different groups (Multiplicity of Infection: MOI = 20). TGF- β 1-LVs transduced MSCs represented the test group and empty vector-LVs served as control group. Engineered MSCs were used in T cells and DCs co-cultures.

T Cell Isolation and Co-Culture with Engineered MSCs. Lymph node T cells were purified by nylon wool from inbred Balb/c mice and the purity was assessed by CD3 staining (>90%). The harvested MSC-TGF- β 1 was co-cultured with T cells (1:10 ratio) for 72 h in RPMI-1640 complete medium. In all wells PHA (1 μ g/ml) was added as general T cells stimulator. Following incubation, T cell proliferation was assessed based on MTT reduction using cell proliferation assay kit I (Roche, Germany). Moreover, the supernatant was collected for assessing IFN- γ and IL-4 by ELISA, according to the manufacturer's instructions (eBiosciences, USA).

Generation of DCs and Co-Culture with Engineered MSCs. Bone marrow cells were obtained from the femur and tibia of female Balb/c mice. Red blood cells were removed by ammonium chloride lysis buffer. Following PBS wash, cells were cultured in RPMI-1640 plus 10% FCS, 20 ng/ml GM-CSF, and 10 ng/ml IL-4. On the third day, the non-adherent cells were re-cultured with 10 ng/ml GM-CSF and 5 ng/ml IL-4 in complete medium till the fifth day that immature DCs (iDCs) were harvested. Engineered MSC-TGF- β 1 cells were co-cultured with derived iDCs (1:10 ratio) for 24 h in RPMI-1640 complete medium, after which DCs harvested, and evaluated for expression of maturation markers, TNF- α secretion, and their potency in allogeneic T cells stimulation. In MSC-DCs co-cultures, control groups included empty vector-MSCs as internal control, iDCs as negative external control, and DCs-LPS as positive external control.

Evaluation of DCs Maturation Markers and TNF- α Secretion. The phenotypes of harvested DCs from MSCs-DCs co-cultures were determined using FACS analysis system. PE-conjugated mAbs against CD40, CD86, MHC-II and PE Cy5-conjugated anti-CD11c were implemented (eBiosciences, USA). The isotype matched control mAbs were also used. Released TNF- α in 24 h cultured DCs were evaluated by ELISA kit (eBiosciences, USA). Tests were done in triplicate.

DCs Allogeneic T Cells Stimulation. To evaluate the functional potency of co-cultured DCs, the lymph node T cells were purified from inbred C57BL/6 mice and then, 2×10^5 T cells and 2×10^4 harvested DCs (irradiated by ^{60}Co -irradiator137) were co-cultured for 72 h in mixed lymphocyte reaction (MLR) test. External negative and positive controls included iDCs (+T cells) and DCs+LPS (+T cells), respectively. Internal control was represented by empty vector-MSCs. After incubation, T cell proliferation was assayed according to cell proliferation assay kit I (Roche, Germany) and IFN- γ and IL-4 culture

supernatants were evaluated by ELISA assays (eBiosciences, USA). Results evaluated from average triplicate tests and three times of repeat.

Statistical Analysis. Data are presented as mean \pm S.D. Statistical analysis of data was performed by one-way ANOVA followed by Tukey's test. P-value of <0.05 was regarded as significant.

RESULTS

T Cells Responses in MSCs-T Cells co-Culture.

T cells were co-cultured with engineered MSCs in different study groups and then after 48 h, T cells proliferation and the amounts of IFN- γ and IL-4 productions were evaluated (Figure 1 A-C). Analysis of the results showed that co-culture of T cells with MSCs caused suppressed proliferation of T cells in all MSCs groups ($p<0.05$). Also TGF- β 1/MSCs significantly suppressed proliferation of T cells in comparison to MSCs and MSCs-LV vector co-cultures ($p<0.05$). TGF- β 1/MSCs cells suppressed IFN- γ release and induced IL-4 production from T cells in comparison to T cells alone ($p<0.05$). Also, IFN- γ suppression of production was more effective by TGF- β 1/MSCs cells in comparison to MSCs and MSCs-LV vector co-culture groups ($p<0.05$). The same pattern of suppression was observed when IFN- γ /IL-4 ratio was compared between groups ($p<0.05$).

Expression of Maturation Markers on DCs Harvested from Co-Culture. After 24 h of MSCs-DCs co-culture, DCs of different groups were harvested and evaluated for the expression surface maturation markers and TNF- α release (Figure 2 and 3). Flowcytometry analysis showed that DCs co-cultured with MSCs (all examined groups) expressed lower levels of CD40, CD80 and MHC-II co-stimulatory molecules in comparison to fully LPS-activated DCs. Comparison of MSCs-DCs co-cultured groups indicated a semi-mature phenotype with lower expression of CD40, CD80 and MHC-II on DCs co-cultured with TGF- β 1/MSCs (compared to MSCs-DCs or MSCs-LV-DCs). The amount of TNF- α release from DCs co-cultured with MSCs (all examined groups) were lower than DCs alone ($p<0.05$). Moreover DCs co-cultured with TGF- β 1/MSCs showed higher suppression of TNF- α release compared to MSCs or MSCs-LV groups ($p<0.05$).

DCs Function in Stimulation of T Cells. DCs potency in T cells stimulation was examined in allogeneic MLR (Figure 4). After 72 h culture of DCs co-cultured with allogeneic T cells, the T cells proliferation and IFN- γ and IL-4 release were evaluated. Results showed that DCs co-cultured with TGF- β 1/MSCs stimulated proliferation of T cells lower than intact DCs and even DCs co-cultured with MSCs or MSCs-LV vector groups ($p<0.05$) (MSCs-LV = $79.4 \pm 3.1\%$ vs. TGF- β 1/MSCs = $70.3 \pm 4.2\%$). Assessment of IFN- γ on MLR cultures showed suppression of IFN- γ release by all MSCs-DCs groups and the most effective suppression observed by DCs interacted with TGF- β 1/MSCs cells ($p<0.05$). IL-4 release was stimulated by DCs co-cultured with TGF- β 1/MSCs cells in comparison to intact DCs and even MSCs-DCs or MSCs-LV-DCs co-cultures ($p<0.05$).

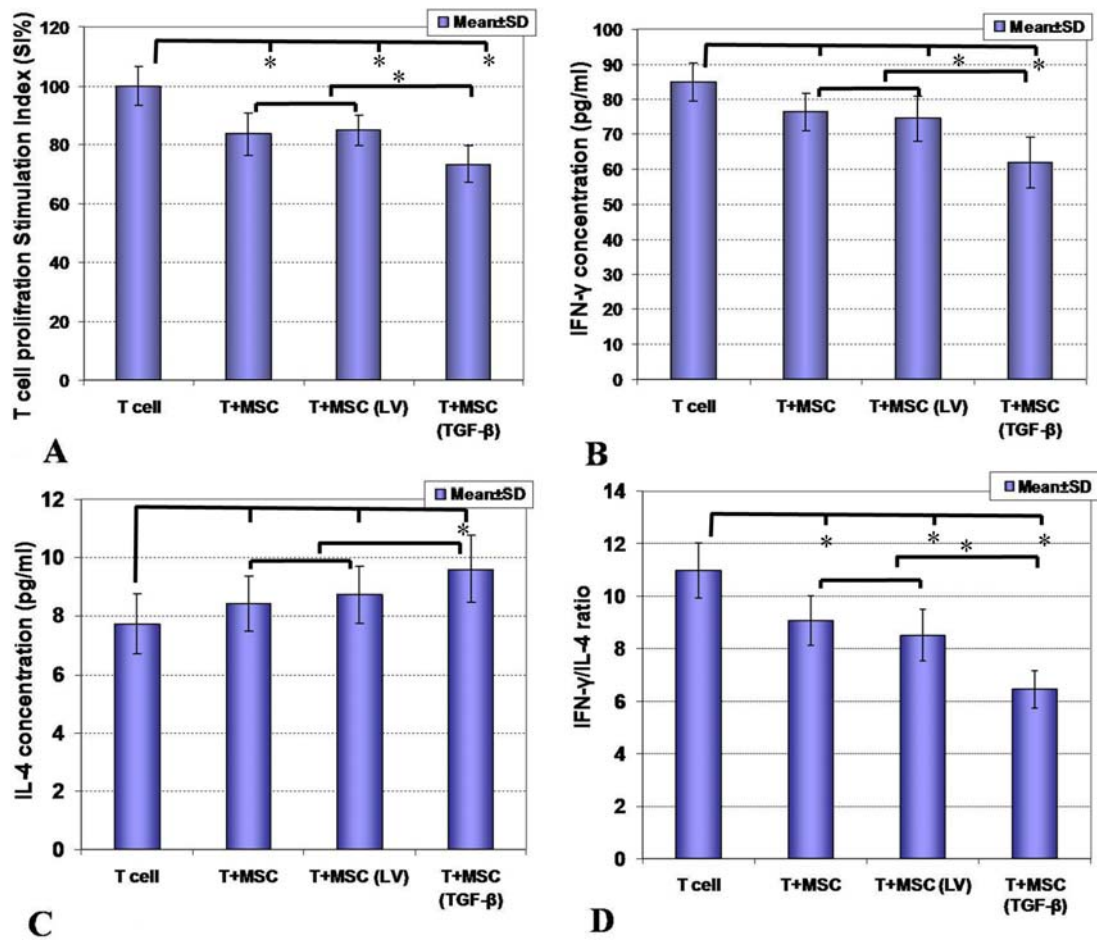


Figure 1. T cells co-cultured with engineered MSCs and T cells proliferation (A), IFN- γ (B), IL-4 (C) secretion evaluated and IFN- γ /IL-4 ratio was calculated (D). Cultures were incubated for 72 hr (in presence of 1 μ g/ml PHA). Statistically significant differences are shown with stars (P<0.05).

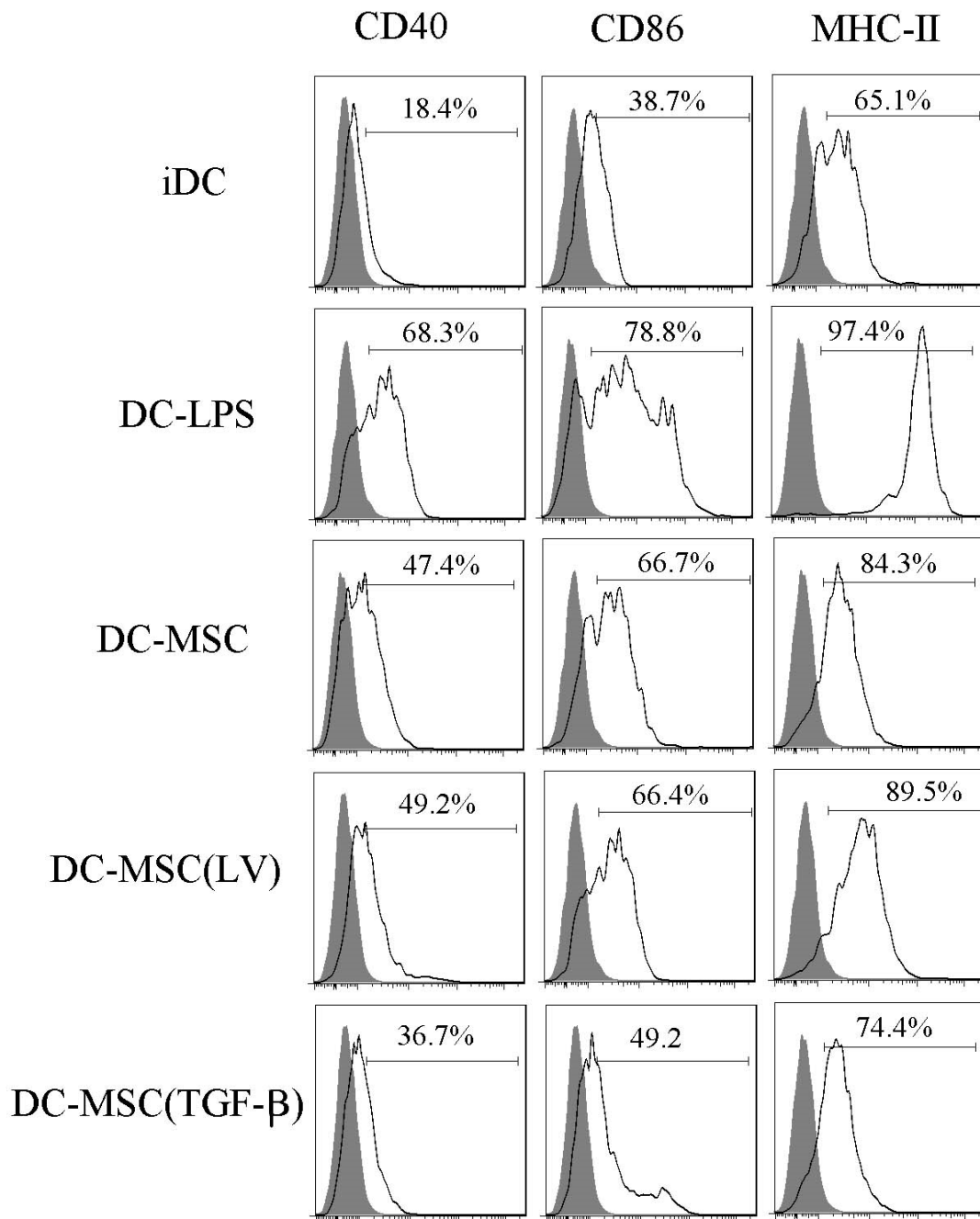


Figure 2. Harvested DCs from MSCs-DCs co-cultures were evaluated for their CD40, CD86 and MHC-II maturation markers expression. While LPS stimulated DCs expressed higher levels of all three markers in compare to iDCs as control groups; DCs co-cultured with MSCs showed lower levels of maturation markers expression in comparison to fully mature LPS-stimulated DCs. DCs co-cultured with TGF- β 1/MSCs showed a semi-mature phenotype. M: Mean Fluorescence Intensity (MFI).

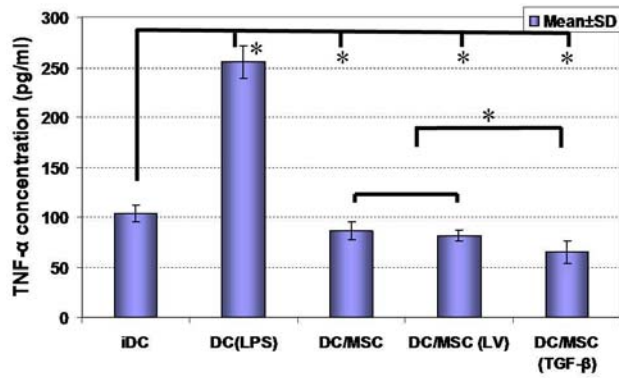
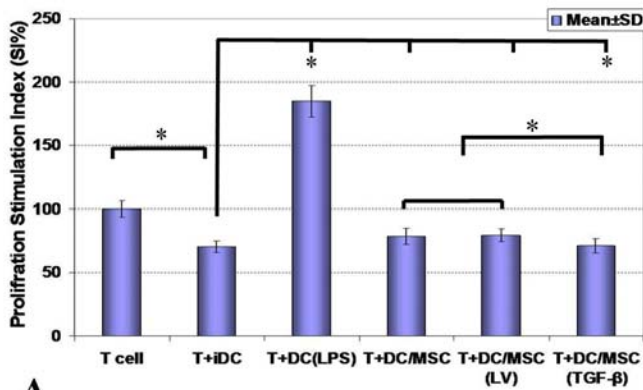
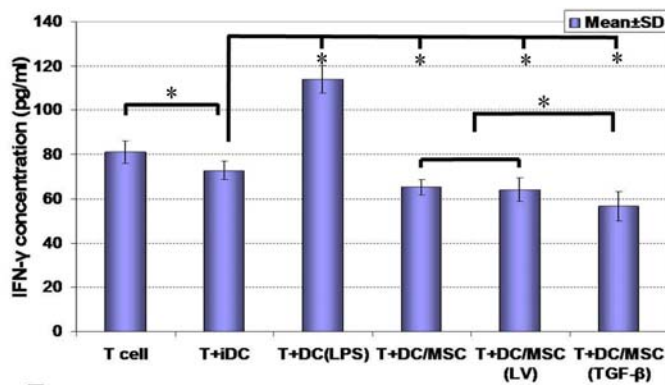


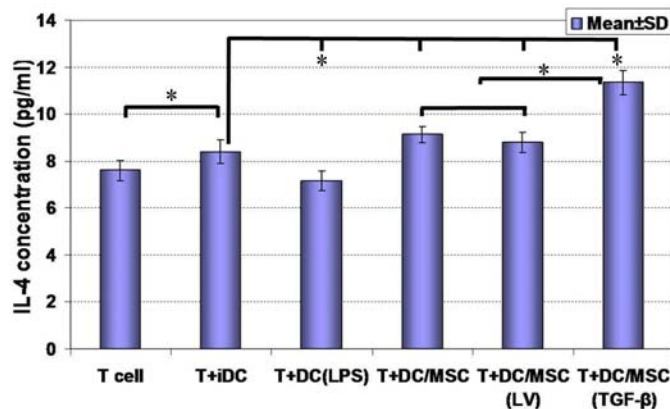
Figure 3. Harvested DCs from MSCs-DCs co-cultures were evaluated for TNF-α secretion. Significant differences between groups are shown with stars (P<0.05).



A



B



C

Figure 4. Functional potency of harvested DCs from MSCs-DCs co-cultures in different groups was assessed by their potency to stimulate allogeneic T cells in MLR test. DCs and T cells (1:10 ratio) cultured for 72 hrs and then T cells proliferation and IFN-γ and IL-4 release were evaluated. Significant differences between groups are shown with stars (P<0.05).

DISCUSSION

The beneficial effects of simultaneous administration of MSCs and TGF- β 1 have been shown in several autoimmune diseases where beneficial functions of TGF- β 1 engineered MSCs in autoimmune diseases are already shown (13-15). A study showed that TGF- β 1 protects MSCs from apoptosis and increases MSCs capacity for nerve regeneration by reduction of inflammation (13). Moreover, adenovirus-TGF- β 1 transfected MSCs could enhance rabbit articular cartilage defects (14). Another study showed that TGF- β 1-gene engineered MSCs, using non-viral vector, can induce cartilage regeneration *in vivo* (15). But, the exact effects of TGF- β 1 engineered MSCs on immune cells have not been clarified yet. In this study we used TGF- β 1 engineered MSCs and evaluated their effects on interaction with DCs and T cells and evaluated cells functions. T cells isolated from syngeneic mice lymph nodes were co-cultured with MSCs in different groups. Results showed that co-culture of T cells with MSCs cause suppression of T cells proliferation; while T cells interacted with TGF- β 1/MSCs demonstrated higher degree of suppression of proliferation in comparison to MSCs alone. The same pattern of suppression was seen in IFN- γ release in co-cultures; but TGF- β 1/MSCs interaction with T cells induced production of IL-4. Numerous immunoregulatory mechanisms of MSC have been described including the secretion of Indoleamine 2, 3-dioxygenase (IDO), PGE2, cytokines or Nitric Oxide (NO). For instance IDO impairs the synthesis of various cellular proteins and leads to inhibition of cell proliferation (16,17). Beside soluble factors, cell-to-cell contact between MSC and T cells can also suppress the function of T cells that acquire regulatory phenotype marked by a sustained expression of CD69, and increased transcript levels of T-reg related genes (18). It has been shown that MSCs can reduce the proliferation of both resting and IL-2 activated NK cells and their cytotoxic capabilities as well as IFN- γ production (3). Also TGF- β 1 inhibits the proliferation of thymocytes, T cells, B cells, and natural killer cells. Lymphocytes and monocytes possess high affinity TGF- β 1 receptors and addition of TGF- β 1 to *in-vitro* cell cultures results in significant modulation of lymphocytes function. TGF- β cooperates with IL-21 to induce CD4⁺ CD25⁺ regulatory Treg cells that counterbalance the effect of IL-6, to promote proinflammatory IL-17-producing T cells. Furthermore, TGF- β inhibits NK cell function through modulation of CD4⁺ CD25⁺ regulatory T cells (19). Therefore, observed suppression of T cells proliferation and cytokines pattern is accompanied by collaborative function of MSCs and TGF- β 1. We should mention that in a contrivers report, Xu *et al.*, reported that TGF- β (10 ng/ml) could promote proliferation of CD3/CD28 activated T cells in the presence of MSCs (20).

In another part of our study, bone marrow derived DCs were co-cultured with TGF- β 1 engineered MSCs. DCs co-cultured with MSCs showed diminished levels of CD40, CD86 and MHC-II maturation molecules in comparison to fully activated DCs. Interaction of DCs with TGF- β 1/MSCs induced a semi-mature phenotype of DCs by moderate expression of CD40, CD86 and MHC-II maturation markers. Moreover, DCs co-cultured with MSCs showed reduced TNF- α secretion and suppression was in higher levels for TGF- β 1/MSCs in comparison with other MSCs groups. It has been shown that MSCs could inhibit DCs antigen (Ag) presentation to CD4⁺ T cells and cross-presentation to CD8⁺ T cells (21,22). In previous studies MSCs significantly reduce the expression of CD83, which is a co-stimulatory receptor for T cell activation, leading to a shift to immature status (21). Moreover, MSC showed diminished expression of

(HLA-DR and CD1a) antigen presenting molecules as well as CD80 and CD86 co-stimulatory molecules and also down-regulated IL-12 secretion. These effects of MSCs are consistent with impaired ability of mature DCs to stimulate the naive T cells (9,23).

In our study, functional potency of DCs also was evaluated by their capacities in stimulation of allogeneic T cells. DCs harvested from MSCs-DCs co-cultures induced lower amounts of T cells proliferation and also IFN- γ secretions and induced IL-4 production from allogeneic T cells with more effectiveness of TGF- β 1/MSCs interaction. MSCs are known to display lymphocytes regulation activities by suppression of lymphocytes proliferation, downregulation of molecules involved in the migration to the lymph nodes or inhibit the production of IL-2 (3,21). Nauta *et al.* showed that the soluble factors of MSCs affect differentiation and the ability of DCs to stimulate naive T cell proliferation (24). MSCs also alter the cytokine secretion profile of DCs. Co-culture with MSCs causes decreased TNF- α release by mature DCs as well as increased secretion of IL-10 by plasmacytoid DCs (9). In another study, Chiesa *et al.* showed that MSCs impair Toll-like receptor-4-induced activation of DCs, affect antigen presentation to CD4⁺ T and CD8⁺ T cells, inhibit secretion of inflammatory cytokines, and down regulate expression of molecules involved in the migration to lymph nodes (23). In case of TGF- β 1, a study showed that TGF- β 1 plays its role in modulation of immune responses (especially through Th17 cells) by its effects on DCs (25). It has been shown that TGF- β 1 inhibits T-cell proliferation and IL-2 (26), suppress Th1 type immune responses (27) and promote Treg and Th17 differentiation and cytokines secretions (28,29). It is shown that TGF- β 1 acts with other inhibitory molecules to maintain a state of tolerance, for instance, TGF- β 1 and CTLA-4 molecules work together to terminate the immune response (10,30). Cooperative functions of MSCs and TGF- β for suppression of immune response are therefore compatible with our results. In conclusion, this study showed cooperative functions of MSCs and TGF- β in form of engineered TGF- β /MSCs that could modulate both T cells and DCs responses. TGF- β 1/MSc cells prevent T cells proliferation and Th1 cytokine secretions. Moreover, TGF- β 1/MSc cells provide a semi-mature phenotype in DCs as leader of immune responses and shift DCs responses to suppressor/regulatory and Th2 patterns. This TGF- β engineered MSCs with carrying suppressive functions of MSCs and TGF- β could be beneficial in clinical conditions such as cell/organ transplant, GVHD and autoimmune diseases.

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