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# Effect of Different Activation Mechanism of T Cells on Immunoregulatory Properties of Bone Marrow Mesenchymal Stem Cells

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## Abstract

***Background:*** *Mesenchymal Stem Cells (MSC) have ability to regulate immune response via proinflamatory cytokines. In this study we aimed to investigate the effect of different mechanism for T cell activation and adjacency on immunoregulatory properties of MSC.*
***Methods****: Bone marrow MSCs were purchased and T cells were obtained from whole blood sample. T cells were activated by two different mechanisms using Peripheral Blood Mononuclear Cell (PBMC) and phytohemagglutinin (PHA) and then co-cultured with MSC using transwell cultures (Indirect contact) and usual plate (Direct contact). After 96 hours TGF-b concentration in culture medium and indole amine 2, 3-dioxygenase (IDO) activity in cell lysate were assayed. We used ANOVA and T-test for statistical analysis and 0.05 was considered as significant level.*
***Results****: Our result showed a significant increase of TGF-b secretion and IDO activity. Increase in mixed lymphocyte culture (MLC) groups was more significant than lymphocyte transformation test (LTT) in compare with control.*
***Conclusions****: The present study confirmed immunoregulatory effect of BM-MSC. Also this study showed that MLC of BM-MSCs and T cells have more immunoregulatory effects than LTT.*

**Keywords:** Mesenchymal stem cell,T cells, Immunoregulatory, TGF-b, IDO.
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## Introduction

Mesenchymal stem cells (MSCs) are found in nearly all tissues of body. MSCs have the potential for self-renewal and differentiating into several cell types. Also in experimental and physiological conditions, MSCs can differentiate into specialized cells.[1,2] there is hope that Stem cells can be used for the treatment of many diseases using cell therapy.[3,4]

MSCs can be obtained from nervous and adipose tissues, bone marrow etc. These cells have a fibroblastic appearance with self-renewal capacity, MSCs can be passaged many times without significant change of distinct properties.[5] Recently, researchers have shown that MSCs have an ability to inhibit immune system activity licensed by inflammatory environment.[6] This property is due to the proinflamatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1 alpha and beta (IL-1) α,β which can stimulate MSCs to elicit very high levels of immunosuppressive factors such as CXCR3 ligand, CCR5 ligand, ICAM1and VCAM1.[7]

We also showed in our pervious study that PBMSC can act as an immune modulator factor via the IL-2 and IFN-γ.[8,9] Mechanism and molecules which mediate MSC-induced immunosuppressive effects are not the same in different species. As an example, iNOs are lightly immunosuppressive at high concentration via undefined pathway in murine model but in human model IDOs are like this.[3,10] In factin response to IFN-γ, MSCs express the major KP enzyme IDO,[11] so they can inhibit T cell proliferation and regulate the function of main cell populations in both adaptive and innate immune systems, including natural killer cells, antigen presenting cells, T- and B-cells.[12] Moreover, IFN-γ-induced activation of IDO in MSCs can create a Tryptophan-depleted milieu which give promotion to immunosuppression.[13] IDO is the first and rate-limiting enzyme in the metabolism of tryptophan. It is produced by many cell types including macrophages, dendritic cells, human MSCs, and most tumor cells,.[14] IDO suppresses immune responses severely by decrease of local tryptophan and increase of accumulation of tryptophan metabolites including quinolinic acid, 3-hydroxyanthranillic acid and kynurenine.[3] However, the actual molecular mechanisms of IDO immunosuppressive function and its overall role in the immune response remain extensively unknown.[3,15]

Family of TGF-b molecules are pleiotropic cytokines with important roles in growth, wound healing immunoregulation and cancer.[15] Three TGF-b isoforms TGF-b1, TGF-b2, and TGF-b3 have been detailed in mammalian species. TGF-b1, the prototype of this family, is a renowned immunosuppressive molecule.[16] Interestingly, TGF-b1 is responsible for MSC-dependent inhibition of T cells proliferation.[17,18]

MSCs modulate immune response via interaction with a diversity of immune cells including dendritic cells, T and B lymphocytes and NK cells using this mechanism. However it is still unclear that cell to cell contact is necessary for starting this mechanism or only environmental cytokines are adequate.[19,20] In this study, we aimed to investigate whether cell to cell contact is necessary for immunosuppressive effects of Bone Marrow MSCs (BM-MSCs) using different activated T-cells.

## Materials and Methods

Protocols and method were performed according our previous study.[8,9]

Human BM-MSCs were purchased as a 70% confluent T75 cell culture flask (Passage 3) from Isfahan Royan Institute. In order to achieve required number of cells for the study, BM-MSCs were expanded in vitro. Purchased cells were immediately cultured in complete DMEM (Gibco, Canada) and 10%FBS (Gibco, Canada). Every two days, cell medium was changed. After 4-5 days cells were detached by trypsin/EDTA solution (Sigma, USA) and again cultured in three flasks. This process was repeated until BM-MSCs got to passage 5 and also adequate count for further co-culture with activated T cells lymphocyte. The cell counting and their viability determination by trypan blue stain were done as standard procedures.[21] All of the culture processes were don in Isfahan University of Medical Sciences.

Five to 15 ml (Depending on the number of co-cultures) heparinized venous blood samples were taken from a healthy person (sample 1) and another healthy allogeneic volunteer (Sample 2).

Isolation of T cell from sample 1: 50µL of RosetteSep® Human T Cell Enrichment Cocktail was added to every 1mL of whole blood and mixed well (was incubated 20 minutes at room temperature).The blood was diluted with an equal volume of PBS+2% FBS and mixed gently. The diluted sample was layered on top of appropriate amount of Ficoll and they were centrifuged for 20 minutes at 2500 rpm at room temperature, with the brake off. After that, the enriched T cells were removed from the Ficoll plasma interface, they were washed with PBS+2% FBS (twice) and were suspended in culture medium.T cell suspension was adjusted at $2\times10^6$ Cells/ml.

Isolation of PBMC from sample 2: 5 ml whole blood diluted with an equal volume of PBS, was gently overlaid on top of 5 ml Ficoll Hypaque and was spined at 2500 rpm for 20 min at RT. The mononuclear cell layer was collected from interphase of PBS/ Ficoll with a sterile pipette and transferred to a new tube. The cells were washed once with PBS (1500 rpm, 10 min) and they were resuspended in RPMI. The cell suspension was adjusted at 2 × 106 viable cells/ml.

Mitomycin treatment of cells:

1- Inactivation of BM-MSC: BM-MSCs were trypsinized, counted and the suspension of $2\times10^6$ viable cells/ml in DMEM medium was prepared and 7µl of Mitomycin stock solution was added to BM-MSC suspension (so final concentration of MMC was 7µg/ml). After 3 hours (in 37 °c), BM-MSCs were washed three times by PBS. This suspension (adjusted to $1\times10^5$ viable cells/ml in completed medium) was prepared for use in co-cultures.

2- Inactivation of stimulator cells: The final concentration of MMC in the cell suspension of PBMCs was adjusted at 20µg/ml. The suspension was set in the incubator for 30 min. Then cells were washed with PBS three times. Again a cell suspension of 2 × 106 viable cells/ml was prepared and used to stimulate T cells in MLC co-cultures.

Co-cultures:

1- Direct co-cultures (in usual plate): Mixed Lymphocyte Culture (MLC): Inactivated BM-MSC + T cell + Inactivated PBMC

At first, to six four-well sets of flat-bottom of 96 well cell culture plates, 100 µl, 40 µl, 20 µl and 10 µl of BM-MSC suspension were added respectively. The volume of cell cultures in all wells was made up to 100 µl with RPMI (completed medium), No BM-MSC was added in four wells (only RPMI 100 µl was added).

The stem cells were allowed to adhere for 18- 20 hours incubation in 37˚C0, 5% CO2. After this time ,one set of four-wells were used as control, so no lymphocytes were added .another set of four- well was chosen for mMlc so only T cells (50 µl) were added to BM-MSC. In another set of four- wells that remained, 50 µl of T cell suspension and 50 µl of stimulator cell suspension (both prepared a few hour before the use as mentioned in below) were added.

In the first set, any kind of antibody was added. A neutralizing anti-human TGF-β1 mAb (at concentration of 5 µg/ mL) was added to second set of four-well. In the third set anti-human TGF-β1 mAb (at concentration 50 µg/ mL) was added. A neutralizing anti-human IFNγ mAb (at concentration 5 µg/mL) was added to the fourth set. Only T cell and PBMC, (as a negative control) separately were added to wells contained culture medium. Also culture medium was as a negative control).Fifty µl of T cell suspension and 50 µl of stimulator cell suspension (PBMC) were added to another well with 100 µl of culture medium alone (positive control. All cultures were done in duplicate. The experiments were performed at least 3 times.[22] The plate was incubated for 96 hour in the incubator.[23]

Lymphocyte Transformation Test (LTT): In these cultures, phytohemagglutinin (PHA; 4 µg/mL) was used instead of PBMC to induce T-cell proliferation. One hundred µl, 40 µl, 20 µl and 10 µl of BM-MSC suspension were added to four-well set of a flat-bottom of 96 well cell culture plate, respectively. (One of four-well sets was used as control, so no lymphocytes were added. With RPMI (completed medium), the volume of cell cultures in all wells were made up to 100 µl. Additionally, to three extra wells, only 100 µl of RPMI (completed medium) were added. The stem cells were allowed to adhere for 18-20 hour incubation in 37˚C, CO2 incubator.

After this time, T cell suspension (prepared at few hour before the use) was diluted with culture medium to obtain the concentration of 106 cells/ml. Amount of 100 µl of this suspension was added to a well contained only 100 µl of culture medium negative control. Then, with PHA stock solution, the final concentration of PHA in T cell suspension was adjusted at 8 µg/ml. One hundred µl of T cell suspension plus PHA were added to four sets of four -wells contained decreasing amount of BM-MSC (like MLC any antibody was added to one of sets but neutralizing anti-human TGF-β1 mAb and IFNγ mAb were added at another set of four- wells, seperately) and a well contained only culture medium. Another one was LTT for positive control. Another four-well set was added 100 µl of culture medium, alone. At the end ,the volume of all wells were made up to 200 µl with culture medium .The plate was incubated for 72 hour in 37˚C, CO2 incubator.

2- Transwell cultures (indirect co-culture): Transwell chambers with a 0.3 µm pore size membrane were used to separate the lymphocytes and stimulators from the BM-MSC. Lymphocytes were co-cultured with inactived PBMC or PHA, whereas BM-MSCs were placed in inner transwell chamber.

The details of doing co-cultures were the same as direct co-cultures, expect that all volumes were increased by 2.5 times.

Measurement of TFG- β1 produced by BM-MSC: Levels of transforming growth factor (TGF)-β1 in BM-MSC culture medium were measured by colorimetric enzyme linked immunoassays according to the manufacturer's instructions. (Quantikine® ELISA Human TGF-β1 Immunoassay, R&D Systems, Inc.USA).

Statistical analysis was performed by the SPSS software and univariate analyse of variance and independent T-test were used to analyze the significance of the mean difference of proliferation between groups. P less than 0.05 was considered to be statistically significant.

## Results

The phenotype of BM-MSC was determined previously by flowcytometery (positive for CD44, CD90, CD29 (stem cell marker) and negative for CD14, CD45and CD34 (hematopoietic marker).

The secretion of TGF-β1 was elevated when MSCs were cultured with MLR and LTT. We detected immunosuppressive cytokines such as TGF-β1 in MSC-conditioned medium. TGF-β1 secretion was elevated when co-cultured BM-MSCs with T lymphocytes stimulated with PBMC or PHA compared with BMSCs single-cultured group. In all ratios, the levels of TGF-β1 was statistically significant (P<0.05) (Data were shown in Table 1 and Figure 1).

The differences between levels of TGF-β1 secretion in co-cultured BM-MSCs with LTT were not statistically significant in comparison with co-cultured BM-MSCs with MLR. The differences between levels of TGF-β1 secretion in co-cultured BM-MSCs with LTT were statistically significant compared with single-cultured group (only BM-MSC, P<0.05). The same results were obtained in co- coultures of BM-MSCs with MLC comparing with only BM-MSCs (P<0.05).

**Table 1. The levels of TGF-β1 secretion in co-cultured BMSCs with T lymphocytes stimulated with PBMC or PHA compared with BM-MSCs single-cultured group**

| BM-MSC/T Cell Ratio | TGF-β1(pg/ml) | | | | |
|---|---|---|---|---|---|
| | T | MLC | LTT | Only BM-MSC | P |
| 1/100 | _ | 5.57 ± 0.31 | 5.01 ± 0.27 | 2.36 ± 0.07 | <0.001* |
| 1/50 | _ | 6.07 ± 0.32 | 5.87 ± 0.26 | 3.53 ± 0.12 | <0.001* |
| 1/25 | _ | 7.73 ± 0.29 | 7.01 ± 0.27 | 4.67 ± 0.10 | <0.001* |
| 1/10 | _ | 9.15 ± 0.30 | 8.90 ± 0.33 | 6.46 ± 0.5 | <0.001* |
| None | 0.71± 0.02 | 1.73 ± 0.20 | 1.53 ± .022 | _ | |

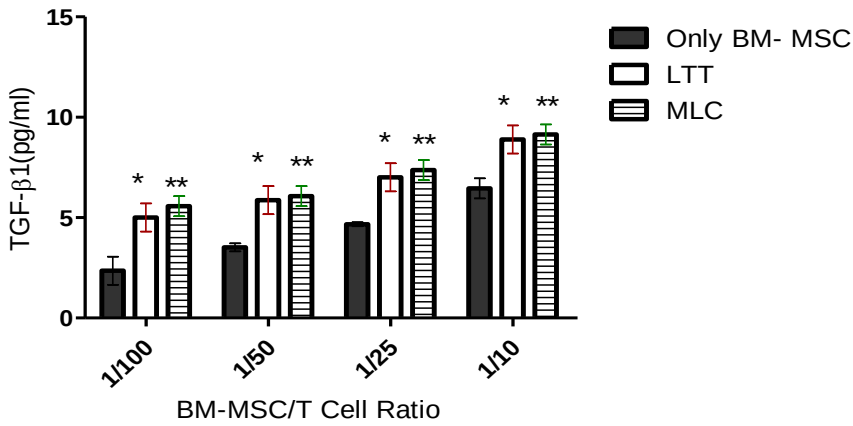


**Figure 1. The levels of TGF-β1 secretion in co-cultured BM-MSCs with T lymphocytes stimulated with PBMC compared with T lymphocytes stimulated with PHA**

## Discussion

Even though it has been widely substantiated that MSCs have potent immunoregulatory functions in vitro and vivo its mechanisms of suppressing alloresponses are not completely understood. This study, explored the role of cell-contact-dependent mechanisms using MLR and LTT models and clarified the contribution of BM-MSC immunosuppressive milieu through cytokine secretion.

A number of studies have suggested that the inhibitory effects of BM-MSCs are mediated by soluble factors, particularly TGF-β1.[15,24] To determine the accuracy of this under our conditions, we cultivated purified human T cells with BM-MSCs at the indicated ratios, in the presence or absence of different dosage of TGF-β1 neutralizing antibody (5μg/ml and 50 μg/ml).

We found that TGF-β1 neutralizing antibody abrogated the inhibitory effect of BM-MSCs on T cells to some extent, however not thoroughly. Addition of anti- TGF-β1 blocking antibody had better influence on reduction of inhibitory activity when used at 10-fold higher concentrations (50μg/mL). These

data support the assumption that TGF- β1plays a major role in MSC-mediated inhibitory activity.

Mauro Krampera et al. suggested that 1µg/mL of TGF-β1 neutralizing antibody had no influence on inhibitory activity of BMSC even when used at concentration 10-fold higher (10 µg/mL).[25]

Di Nicola et al showed that the induced suppression of responder T cells by human MSCs against stimulator PBMCs could be abrogated by high concentrations of neutralizing antibodies versus hepatocyte growth factor (HGF) and TGF-β1. Blocking of each factor separately resulted in a minimal effect on suppression, inasmuch as neutralization of the cytokines simultaneously restored all proliferation of T cells.[26]

These studies may not be similar with our study because Di Nicola[26] and co-workers used blocking antibody in lower dosage and Krampera[26] worked in another Species (mice) which could explain these different findings.

Also in this study, the levels of TGF-β1 were determined in the conditioned media of T cell, MLR and LTT, both in the BM-MSCs cultured alone or presence/absence of BM-MSCs. TGF-β1was detected in conditioned media of both BM-MSCs and immune cells. Results indicated that the TGF-β1 secretion was elevated when co-cultured BMSCs with T lymphocytes, compared with BMSCs single-cultured group, indicating that the interaction of immune cells with BM-MSCs result in increased TGF-β1secretion.

This work emphasizes that effect of superior inflammatory cytokines at the site of transplantation and anti-inflammatory cytokines secreted by MSC should be considered for accessing the immunomodulatory behavior of these cells in different clinical settings. Tissue damage and inflammation are co-localized processes and coincident. Hence it is not unexpected that MSC can be activated by inflammatory signals and such activation should support up-regulation of potent immunomodulatory activity. Therefore the current study reinforces the potential useage of MSCs in the clinical setting for inflammation characteristic.

In the current study, we confirmed which human MSC via the action of TGF-b upregulated the intracellular enzyme of IDO and so inhibition of IDO activity resulted in a whole abrogation of the immunosuppressive effect of MSC on T cells. In our previous study we showed that MSCs can inhibit T cell proliferation by increasing of IFNγ and IL-2.[8,9]

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## Conflict to Interest

The authors declare that they have no conflict of interest.

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