

Comparing the Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth and Bone Marrow-derived Mesenchymal Stem Cells

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ABSTRACT

Stem cells from human exfoliated deciduous teeth (SHED) have been introduced recently and possess characteristics similar to mesenchymal stem cells (MSCs). Because of their convenient accessibility and safety of harvest, SHED can be a preferable source for the ever-increasing MSCs' applications. While they are new, their immunoproperties have not been adequately studied. In this study, we aimed to explore the effect of SHED on T lymphocytes and compare it to conventional MSCs (BMMSCs).

At first the isolated T lymphocytes were activated specifically/nonspecifically *in vitro* and cocultured with SHED or BMMSCs under the same conditions, subsequently their proliferation and cytokine secretion (IL-2 and IFN- γ) were measured.

In our experiment, BMMSCs and SHED inhibit the proliferation and cytokine production of both PHA and alloantigen stimulated T lymphocytes in a dose-dependent manner. In direct and indirect contact to T lymphocytes, the inhibition of BMMSCs (but not of SHED) was significantly different. The cytokine production from activated T cells was affected differently by two types of MSCs. The inhibition decreased by the separation of lymphocytes and MSCs by a semipermeable membrane, but it was not abolished.

This study showed that SHED suppress the activation of human T lymphocytes *in vitro* like other MSCs. Compared to BMMSCs, this suppression was alleviated. In the equal conditions, the pattern of immune-modulation of BMMSCs and SHED was different, suggesting that SHED do not exert the exact mechanisms of BMMSCs' immunosuppression. This finding should be verified by further studies focused on the detailed mechanisms of the immunomodulation of SHED and also BMMSCs.

Keywords: Cytokine; Immunoregulatory; Mesenchymal stem cells; Stem cells from human exfoliated deciduous teeth (SHED); T lymphocyte

INTRODUCTION

The stem cells are undifferentiated cells, with a high capacity of proliferation which can differentiate into many kinds of differentiated cells as well as renew their own population.^{1,2} On account of these distinctive properties, they have been the focus of new medical researches for novel clinical applications, such as regenerative medicine and cellular therapy over the last decades.^{3,4}

Amongst the several types of stem cells,⁵ a specific type of adult stem cells (ASC) called mesenchymal stem cells (MSC) attract most attention.^{6,7} Beside the stemness, MSCs have extra intriguing features, including supporting hematopoiesis, tissue remodeling/repair properties, and angiogenesis.^{8,9} However, none of these properties is the main reason of the popularity of MSCs in current medicine, but it is their unique immunomodulatory property. In the field of stem cell-derived therapies, numerous reports show that stem cell therapy triggers lymphocyte infiltration that result in immune rejection of the stem cell transplant.⁵ On the other hand, there is convincing evidence that MSC escape immune recognition; thus evading the further immunological rejection. They also modulate immune responses via interaction with a variety of immune cells including T and B lymphocytes, dendritic cells (DCs) and natural killer cells (NK).^{10,11} These unique immunomodulatory capabilities expand the potential clinical applications of MSCs to the novel immunosuppressant therapies (e.g. prevention and treatment of rejection after hematopoietic stem cell and solid organ transplantation, treatment of autoimmune or inflammatory diseases).^{12,13}

During the recent years, reparative and immunomodulatory properties of MSCs have been rather successfully tested in a variety of animal models. As a result they have been rapidly applied in human clinical trials.^{14,15} Overall, the promising results of several preclinical and clinical studies^{16,17} anticipate a vast therapeutic application of MSCs in the near future. However, an important issue is the suitable tissue sources from which MSCs can be achieved by convenient, the least cost, side-effects and ethical hurdles.^{18,19}

Nowadays, there is a standpoint to find alternative sources for MSCs, other than the conventional one,²⁰ bone marrow (BM); along with the inaccessibility of BM, obtaining MSCs from it is difficult and obligated an invasive procedure. Moreover, MSCs are relatively

rare (just about 0.001 to 0.01%) in BM and their numbers and differentiation capacity considerably declines with age.^{19,21} Moreover, some current data indicate that bone marrow stem cells may contribute to cancer development.²²

Fortunately, soon after the first characterization of MSCs in the bone marrow,⁷ a flurry of further studies proved that several other tissues contain MSCs as well.²³ Up to now, these cells have been isolated more easily from diverse tissues that some of them are more accessible than BM, such as circulating blood, spleen, amniotic fluid, cartilage, placenta, adipose tissues, fetal tissues, thymus, umbilical cord and either permanent or deciduous dental pulp.^{6,24}

Among these sources, the exfoliated deciduous dental pulp may be an attractive source of MSC. Exfoliated deciduous teeth are readily accessible in large numbers, thus represent a vast reservoir of MSCs. They are discarded as a biological waste, so are free from ethical concerns. Moreover, it is an easy process to obtain stem cells from human exfoliated deciduous teeth (SHED).²⁵ Besides all, SHED are MSCs with a more proliferative capacity than those derived from BM. These valuable features have even encouraged some researchers to propose the idea of setting up a SHED bank.²⁶

Despite these advantages, because SHED compared to other MSC populations such as BM-MSC (bone marrow derived MSC), adipose derived stem cell (ADSC) and umbilical cord blood derived MSC (UCB-MSC) are rather new,²⁷ they are less-studied and less information in particular about their immune properties, are available in the literature. Since an increase in our understanding of SHED immunoregulatory will offer an insight into the use of these cells in human therapy, further investigations in this field is necessary, hence in this work the effect of SHED on activated T lymphocytes *in vitro* were explored and then compared to the effect of BM-derived MSCs as the control.

MATERIALS AND METHODS

In Vitro Expansion of Human Stem Cells

One T25 cell culture flask of SHED (passage 2) was kindly provided from *Torabi negad Research Center (Dental School, Isfahan University of Medical Sciences)* for this research. These cells had been isolated from extracted pulp tissues of normal exfoliated deciduous teeth of six- to nine-year-old

Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth

children as instructed by Huang et al.²⁸. SHED were detached by trypsin/EDTA solution (Sigma) and cultured in complete DMEM (low glucose DMEM medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Gibco) and 1% penicillin/streptomycin (Roche)), in 75cm³ cell culture flasks for further passages to be expanded. Approximately, every two days, the medium of cells was changed. Every four to five days, when cells reached more than 80% confluency, were again trypsinized and subcultured for more passages. This process was repeated until SHED got to passage 4 and a sufficient amount of them was collected for further cocultured with lymphocytes.

Human BM-MSCs were purchased as a 70% confluent T75 cell culture flask (passage 3) from Isfahan Royan Institute. In order to obtain required number of cells for cultures with lymphocytes, BM-MSCs were also expanded *in vitro* as SHED.

The expansion of both human stem cells and all of the cocultures explained below were done in Cell culture lab of Department of Anatomical Sciences and Molecular Biology in Medical School, Isfahan University of Medical Sciences.

Isolation of Human T Lymphocytes

T Lymphocytes were isolated by negative selection from buffy-coats of a healthy volunteer, using RosetteSep® Human T Cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC, Canada). The Lymphocytes were suspended in complete RPMI1640 (RPMI1640 with L-glutamine (Sigma) supplemented with 10% FCS (Gibco) and 1% penicillin/streptomycin (Roche)).

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were obtained on Ficoll density gradient by centrifugation from heparinized peripheral blood samples, obtained from healthy volunteer donor (that was allogenic to person who donated T lymphocytes). The isolated cells then were suspended in complete RPMI1640 .

Cocultures

To study the effect of both types of human MSCs on the activation of human T lymphocytes, they were separately cocultured in different numbers with either mitogen (phytohemagglutinin (PHA)) or alloantigen (allogenic PBMCs) stimulated T lymphocytes as

below. The proliferation and cytokine secretion of T cells were assayed.

Direct Cocultures

Both types of human MSCs were mitomycin inactivated (incubation for 3 hrs. at 7.5µg/ml) to prevent their proliferation. Then, cell count and viability were assessed by trypan blue dye exclusion. Cells were cultured as follows: aliquots of 100µl complete RPMI1640 containing diminishing number (10⁴, 4×10³, 2×10³ and 10³ cell) of MSCs (SHED and BM-MSCs, separately) were plated in flat-bottomed 96-well plates and were allowed to adhere to the plate for 12 to 16 hr. In some wells complete RPMI1640 without MSCs, for controls were added.

After this time, for the mitogen proliferative assay (lymphocyte transformation test, LTT), 10⁵ T cells - stimulated by a non-specific mitogen (4 µg/ml phytohemagglutinin (PHA; Roche)) -in 100µl complete RPMI 1640, were added to MSCs. For control cultures, in a well contained complete RPMI 1640 without MSCs, 10⁵ PHA-stimulated T cells (as positive control) and in another similar well, 10⁵ unstimulated T cells (as negative control) in 100µl complete RPMI 1640 were added. Additionally, to a set of wells contained diminishing number (10⁴, 4×10³, 2×10³ and 10³ cell) of MSCs (SHED and BM-MSCs, separately), only 100µl complete RPMI1640 (without T cells) were added (as background controls). The cultures incubated for 3 days at 37°C in a humidified atmosphere of 5% CO₂.

In the mixed lymphocyte cultures (MLCs), after the adherence of MSCs (SHED and BM-MSCs) to the plate, responder cells (10⁵ T cells) in 50µl complete RPMI1640 were added to MSCs and then an equal number of mitomycin inactivated PBMCs (25µg/ml for 40 minutes) in another 50µl complete RPMI1640 as stimulator cells were added to them. Control cultures were performed in two wells which contained complete RPMI1640 without MSCs: for positive control responder plus stimulator cells were added and for negative control only responder cells were added. Moreover, for modified MLCs, only responder cells (unstimulated 10⁵ T cells) were added to MSCs. Cultures as background controls (cultures of different number (10⁴, 4×10³, 2×10³ and 10³ cell) of MSCs, without lymphocytes) also were performed as in LTTs. Finally the cultures incubated for 5 days at 37°C in a humidified atmosphere of 5% CO₂.

Indirect Cocultures

To study the need for cell– cell contact in inhibition, transwell analysis was performed using a 24-well transwell insert system from Costar (the two chambers of each well were separated by a semipermeable membrane with a pore size of 0.4 μm). The lower chambers contained different number (2.5×10^4 , 10^4 , 5×10^3 and 2.5×10^3 cell) of inactivated MSCs (SHED and BM-MSCs, in separate wells). For LTTs, 2.5×10^5 PHA-stimulated human T cells were cultured in the upper chamber and for MLCs 2.5×10^5 T cells plus an equal number of mitomycin inactivated PBMCs in the upper chamber of wells were cultured. In each case appropriate control, including positive, negative and background controls also were done. All transwell cultures were performed in a total volume of 500 μl of complete RPMI 1640 medium.

After incubation time (3 days for all LTTs and 5 days for all MLCs), T lymphocyte proliferation and cytokine secretion were assessed.

Proliferation Assays

T cell proliferation was evaluated by measuring the incorporation of Bromodeoxyuridine BrdU (a thymidine analogue) into the DNA of proliferating T cells, using a Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche); BrdU was added to the pellet of each well 20 hours before the end of culture time. For the analysis of cytokines, first 150 μl of supernatant was removed from each triplicate well after centrifugation of the culture plate. Thereafter, incorporated BrdU in proliferating T cell was detected by immunoassay according to manufacturer protocol of the kit.

All cultures were established in triplicate and the whole of experiments were done twice.

Cytokine Analysis

The amount of IL-2 and IFN- γ were assayed in pooled supernatant of three repeat of each culture sample, using a Human IL-2 ELISA Kit and Human IFN- γ ELISA Kit (both from R&D System), respectively.

Statistical Analysis

The statistical analysis was performed using SPSS software. Statistical significance was calculated using *t* test analyses and Univariate analyse of variance. Significance was set at $p < 0.05$ (*).

RESULTS

To obtain the accurate proliferation (the pure absorbance) of T cells in the cocultures, the absorbance of each background control was subtracted from corresponding cocultures and to compare T lymphocyte proliferation in different cultures, stimulation index (SI) values were calculated by the following formula:

SI = proliferation of stimulated T cells with or without MSCs/proliferation of unstimulated T cells alone.

Finally, the percentage of inhibition was calculated as follows:

Inhibition = $100 - (\text{proliferation of intended culture} / \text{proliferation of corresponding positive control}) \times 100$.

All values were expressed as the mean and SEM (standard error the mean).

Both Types of MSCs Suppressed the Proliferation of T Cells

The calculated stimulation indexes (SI) for mitogen/alloantigen activated T cell cultures in the presence of different numbers of MSCs (SHED and BMMSC), as well as inactivated T cell cultures in the presence of MSCs (SHED and BMMSC) are depicted in Figure 1.

Generally, the presence of MSCs (whether SHED or BM-MSC) resulted in a statistically significant decrease in PHA/alloantigen-induced proliferation of T lymphocytes (Figure1 and Table 1).

Neither BM-MSCs and nor SHED, themselves did not elicit the proliferation of T cells (Figure1). Also both type of MSCs (BM-MSCs and SHED) significantly impaired T cell proliferation in a dose-dependent manner (more numbers of MSCs, more decrease in T cell proliferation) and these results repeated when LTT and MLC cultures were considered separately (Table 1).

BM-MSCs and SHED Suppressed T Cell Proliferation in Direct and Indirect Cultures Differently

The Inhibition of activated T cells in usual cultures (direct contact to MSCs) and transwell cultures (indirect contact to MSCs) are shown in Figure 2. The mean of Inhibition was statistically different between direct and indirect cocultures of stimulated T cells with BMMSCs, but in general, no significant difference

Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth

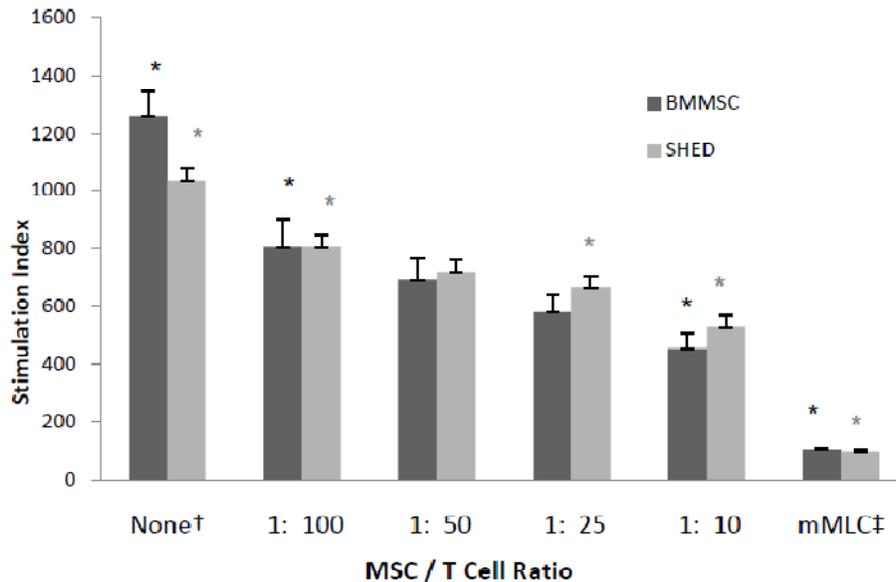


Figure 1. Proliferation indices decreased by the increase in the number of MSCs. These data refer to stimulated T lymphocytes overall (without regard to the type of stimulation). The data are shown as Mean ± SEM. The star (*) represents the difference of a group compared to previous one if statistically significant.

†None: The cultures of allogenic PBMCs / PHA-stimulated T lymphocytes without MSCs, including positive control cultures.

‡mMLC: Coculture of MSCs (SHED and BMMSCs, individually) with unstimulated T lymphocytes at 1: 10 ratio.

was revealed between the mean proliferation of T cells in direct and indirect contact to SHED ($p=0.661$).

In Figure 3, total calculated Inhibition of T cell proliferation for each type of MSC; BM-MSCs and

SHED, in all cocultures were shown. When MSCs were cocultured with stimulated T lymphocytes, under equal circumstances BM-MSCs represented a more powerful suppressive effect on activated T cells.

Table 1. Inhibition of proliferation in the T cell cultures with different number of MSCs. The table illustrates the Mean ± SEM of Stimulation Index of T lymphocytes activated specifically (by alloantigen) and also non-specifically (by mitogen). Regardless of the way of activation, by decreasing the number of both MSCs, T cell proliferation was increased. However, the differences between two tandem ratios were not always statistically significant. For example in LLT cocultures of SHED, the inhibition at 1:100 ratio decreased significantly rather to other ratios, but the differences between 1:50, 1:25 and 1:10 ratios were not statistically significant. The star (*) represents that the difference between the groups (especially each group to control/none) is statistically significant.

MSC/T Cell Ratio	Inhibition			
	BM-MSC		SHED	
	LTT	MLC	LTT	MLC
1: 100	30.084 ± 4.938	45.070 ± 3.300	8.959 ± 0.838	17.083 ± 0.610
1: 50	41.863 ± 3.774	49.662 ± 3.261	27.302 ± 1.665	20.240 ± 2.598
1: 25	51.709 ± 2.996	57.443 ± 3.111	27.908 ± 0.695	31.0004 ± 3.949
1: 10	61.496 ± 3.086	67.129 ± 2.835	34.083 ± 4.811	45.932 ± 7.406
none†	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
<i>P value</i>	0.00*	0.00*	0.00*	0.00*

†None: The cultures of allogenic PBMCs / PHA-stimulated T lymphocytes without MSCs, including positive control cultures.

Table 2. Cytokines decreased with the increase number of both MSCs. The Mean \pm SEM of both cytokines for two sets of experiment are represented here. The star (*) indicates that the difference between the groups (especially each group to control/none) is statistically significant.

MSC/T Cell Ratio	Cytokine Production			
	BM-MSC		SHED	
	IFN- γ	IL-2	IFN- γ	IL-2
mMLC‡	4.823 \pm 0.0410	2.569 \pm 0.063	11.99 \pm 0.20	16.1 \pm 0.931
1: 100	55.863 \pm 9.380	59.345 \pm 4.516	45.45 \pm 5.45	79.494 \pm 9.900
1: 50	60.062 \pm 8.079	49.772 \pm 3.199	31.69 \pm 5.22	55.460 \pm 9.282
1: 25	56.430 \pm 9.442	42.613 \pm 1.586	23.5 \pm 3.13	36.693 \pm 5.595
1: 10	52.077 \pm 6.306	40.636 \pm 1.776	16.56 \pm 2.15	22.077 \pm 3.669
Only T Cell	0.0066 \pm 0.003	0.277 \pm 0.062	4.437 \pm 0.970	3.891 \pm 0.504
none†	102.245 \pm 0.763	112.744 \pm 10.673	86.35 \pm 7.23	163.030 \pm 29.145
P value	0.00*	0.00*	0.00*	0.00*

†None: The cultures of allogenic PBMCs / PHA-stimulated T lymphocytes without MSCs, including positive control cultures.

‡mMLC: Coculture of MSCs (SHED and BMMSCs, individually) with unstimulated T lymphocytes at 1: 10 ratio.

MSCs Reduced the Production of Cytokines from Activated T Cells

The amount of two T cell produced cytokines, IL-2 and IFN- γ in all experiments was measured by sandwich ELISA (R&D System) in pooled supernatant of three repeat of each coculture. Table 2 represents

assayed amounts of IL-2 and IFN- γ in mitogen/alloantigen activated T cell cultures in the presence of different numbers of MSCs, as well as unstimulated T cells alone. As it is observed, there was a significant decrease in the amount of IL-2 and IFN- γ produced by activated T cells, in the presence of MSCs.

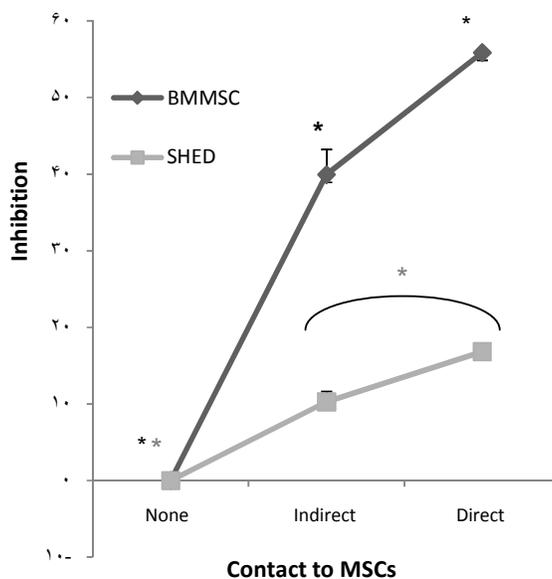


Figure 2. Inhibition of proliferation of T cells in the usual and transwell cocultures. Alloantigen/PHA-stimulated human T lymphocytes were cultured in direct or indirect contact to MSCs (SHED and BM-MSCs, individually). -The data are shown as Mean \pm SEM. The inhibition of T cells diminished considerably when they were separated from BM-MSCs. However, T cell inhibition was not statistically different when they were in direct or indirect contact to SHED. The star (*) represents $p < 0.05$.

Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth

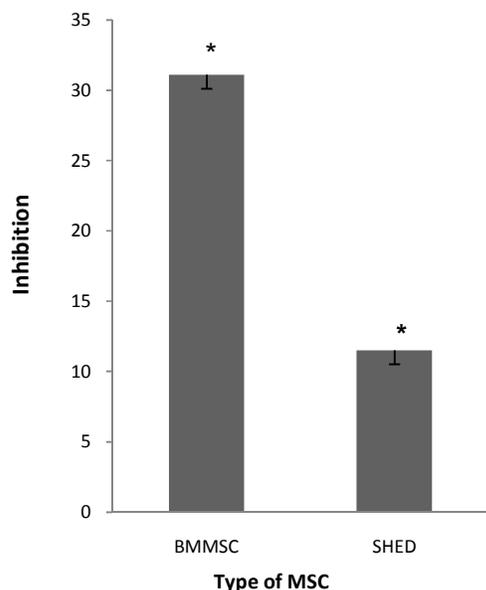


Figure 3. BMMSCs suppress T cells more effectively than SHED. While both SHED and BM-MSCs inhibited the activation of stimulated human T lymphocytes, but in the same experimental conditions, SHED-mediated immunosuppression was significantly less than BM-MSCs.

When the specific and non-specific ways of activation of T lymphocytes were considered separately, the decrease in the amount of IL-2

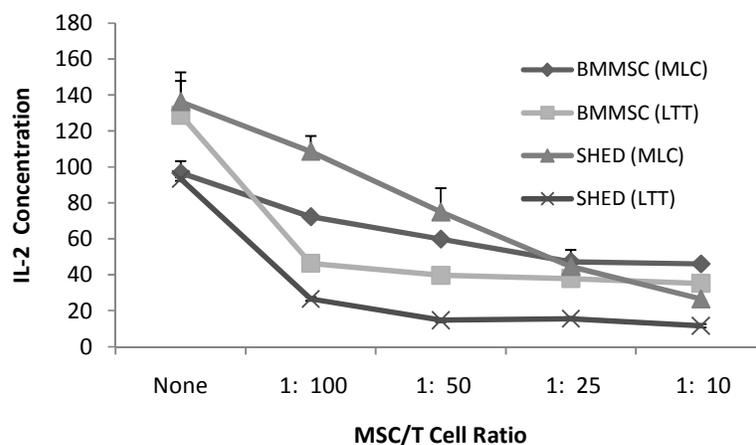


Figure 4. The assayed IL-2 in LTTs and MLCs with different number of MSCs. The detected amount of IL-2 was reduced by the increased number of MSCs (whether SHED or BM-MSCs). But this was not statically significant about LTTs of BM-MSCs and also LTTs of SHED. The data are shown as Mean \pm SEM for two sets of experiments.

by the increase in the number of both MSCs was seen. But that was not statistically significant in LTT cocultures of both BM-MSCs and SHED ($p=0.432$ and $p=0.368$ respectively) (Figure 4).

The detected amount of IFN- γ in alloantigen-stimulated and also PHA-stimulated T lymphocytes cocultured with BM-MSCs and or SHED decreased significantly with the increase in the numbers of MSCs (Figure 5). However, in LTT cocultures of SHED, when the number of SHED increased, the decline in the amount of IFN- γ was not statistically significant ($p = 0.403$) (Figure 5).

MSCs Influence the Production of Cytokines from Activated T Cells in Direct and Indirect Contact

The assayed amounts of IFN- γ and IL-2 in usual and transwell T cell cultures are illustrated in Figure 6. Data analysis revealed a significant reduction of measured amount of IFN- γ , when T lymphocytes were cocultured in transwell plates (indirect contact) with either SHED or BM-MSCs. Direct cultures of SHED with stimulated T cells decreased IFN- γ amount, significantly. In contrast, detected decline in the assayed IFN- γ in cocultures which T cells were in direct contact to BM-MSCs was slightly and not statistically significant (Figure 6A).

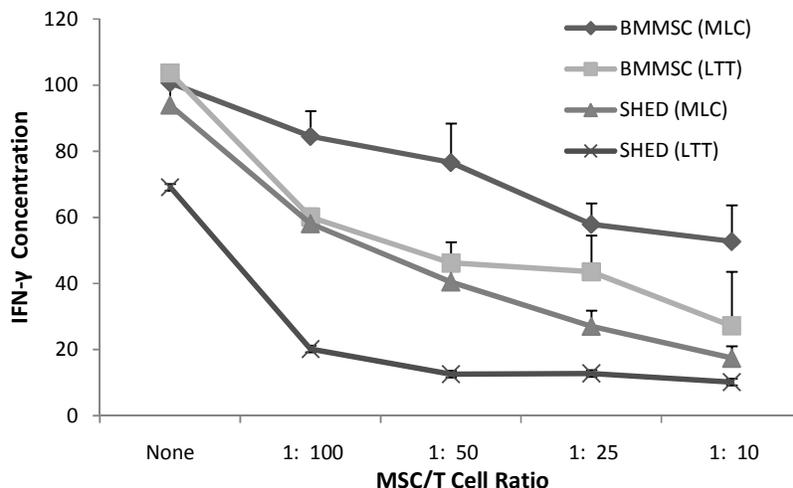


Figure 5. The assayed IFN- γ in LTTs and MLCs with different number of SHED or BM-MSCs. The amount of IFN- γ diminished by the increase in number of both MSCs. However, this reduction was not significant for LTTs of SHED. The data are shown as Mean \pm SEM for two experiment sets.

The detected amount of IL-2 cytokine in cultures of activated T lymphocytes in the presence of MSCs (whether SHED or BM-MSCs) showed a significant reduction. However, the separation of MSCs (either SHED or BM-MSCs) from T cells by a permeable membrane increased the assayed IL-2 in respect to direct contact, but not significantly (Figure 6B).

Although, the amount of IL-2 represented no statistically significant difference between direct and indirect LTT cocultures of both MSCs and also in MLCs of BM-MSCs (Table3), but the separation of

SHED from T cell by a permeable membrane in MLCs resulted in the statistically significant increase of the amount of IL-2 (Table 3).

In the case of IFN- γ , when the contact state was changed from direct to indirect, for both culture types (MLCs and LTTs) of BM-MSCs as well as MLCs of SHED, the differences in detected IFN- γ were statistically significant. However, the increase in the assayed IFN- γ for indirect LTTs of SHED was slight and not significant (Table 3).

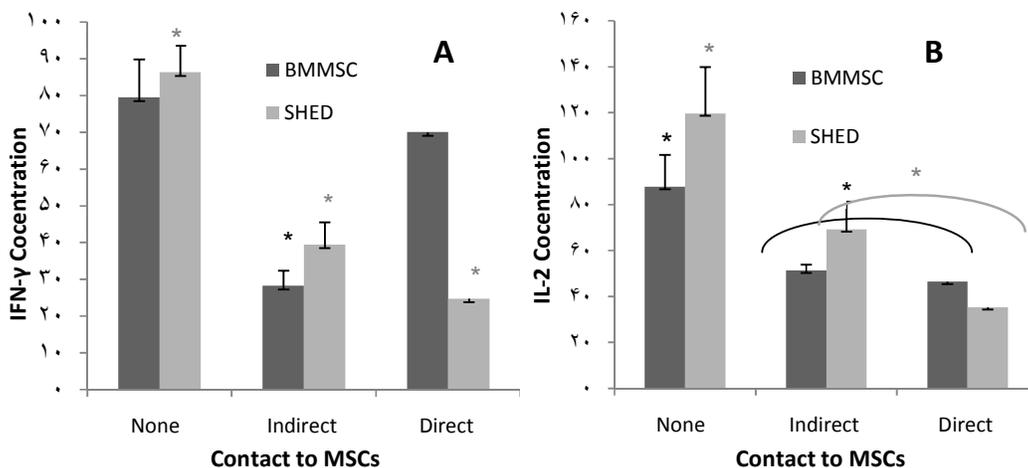


Figure 6. The amount of IFN- γ (A) and IL-2 (B) produced by stimulated T cells in coculture. The Mean \pm SEM of both cytokines for two sets of experiments are represented here. The star (*) represents $p < 0.05$ and show that the difference of the intended group is statistically significant compared to other groups.

Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth

Table 3. Cytokine concentrations in direct and indirect of both type of cocultures (LTT and MLC) of MSCs (BM-MSCs and SHED) and activated T cells. The table illustrates the Mean \pm SEM of ELISA-detected amount of IL-2 and IFN- γ produced by T lymphocytes activated specifically and also non-specifically which were cocultured in direct/ indirect contact to MSCs. The star (*) represents that the difference between two intended groups is statistically significant.

Contact	IFN- γ Secretion				IL-2 Secretion			
	BM-MSC		SHED		BM-MSC		SHED	
	LTT	MLC	LTT	MLC	LTT	MLC	LTT	MLC
Direct	57.625 \pm 5.427	82.438 \pm 3.4862	12.905 \pm 0.644	23.215 \pm 3.056	36.443 \pm 0.573	56.505 \pm 3.034	7.582 \pm 0.360	49.208 \pm 6.282
	17.543 \pm 2.943	38.977 \pm 5.646	15.490 \pm 2.356	53.465 \pm 6.865	46.606 \pm 3.276	56.043 \pm 3.420	31.431 \pm 4.810	107.062 \pm 13.734
<i>P</i> value	0.00*	0.00*	0.471	0.00*	0.076	0.945	0.513	0.00*

DISCUSSION

Shi and colleagues in 2003 reported the isolation of stem cells from human exfoliated deciduous teeth (SHED).¹ Subsequent researches established that they possess characteristics similar to MSCs.^{25,30,31} Because of their convenient accessibility, safety of harvest, being ethically uncontroversial, SHED can be a preferable source for the ever-increasing MSCs' application in experimental and preclinical settings and likely for future stem cell-based therapies.^{25,31,32} While they are new, compared to other MSC populations, their immune properties have not been studied yet, as much as necessary. In this study, we explored the effect of SHED on T lymphocytes as the chief executives of the immune response and compared to conventional BM-MSCs. With this aim, the isolated T lymphocytes from a volunteer were activated both specifically and nonspecifically *in vitro* and cocultured with SHED or BM-MSCs under same conditions, and then their proliferation and cytokine secretion (two indicators of T cell activation) were measured.

The first point in immune properties of MSCs is their immune-privileging which has been reported *in vitro* by numerous researchers as failure of various MSCs to activate resting lymphocytes and induce them to proliferate.^{33,34} MSCs exhibit low expression of major histocompatibility complex (MHC) class I molecules, and are negative for MHC class II antigens.³⁵ Moreover, MSCs do not express costimulatory molecules such as B7-1 (CD80), B7-2 (CD86), CD40, and CD40 ligand and therefore, they escape immune response.^{11,36} In our experiment, neither BM-MSCs nor SHED cocultured with responder T

cells (in modified MLCs) could elicit the proliferation of allogeneic T cells, indicating the low immunogenicity of both.

In addition to the immune evasion, MSCs have a potent capacity to inhibit the activation of immune cells;³⁷ abundant reports have demonstrated that MSCs are able to suppress an ongoing immune response by inhibiting the stimulated T-cell proliferation; whether these T cells were stimulated by mitogens or by alloantigens and whether they were in physical contact to T cells (direct contact, in usual plates) or were separated from T lymphocytes by a permeable membrane (indirect contacts, in transwell plates).^{34,38-40} According to our results, BM-MSCs as previously have been demonstrated⁴¹⁻⁴³ and also SHED, similar to MSCs from other sources such as periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs),⁴⁴ placenta-derived MSC,^{33,39} ADSC^{38,45} and UCB-derived MSC⁴⁶ could inhibit the proliferation of both PHA-stimulated T lymphocytes and T cells activated by allogeneic PBMCs.

On the other hand, there are a few studies that have displayed MSCs as immunostimulatory cells that enhance immune reactions and may function as antigen presenting cells (APC) and induce T cell responses⁴⁷⁻⁴⁹ It is notable that even in these studies, MSCs were not merely immunostimulator but they increased the proliferation of stimulated lymphocytes only at a certain ratio range.⁴⁸⁻⁵⁰ Additionally, studies of Chan,⁵¹ Stagg,³⁵ and Schurgers⁵² have demonstrated that the presence of different amounts of some cytokines affect the immune properties of MSCs in completely opposite ways. Besides, other investigations showed that trigger the same receptors (some Toll-Like Receptors) on MSCs

may enhance^{53,54} or diminish^{54,55} the immunoregulatory functions of MSCs depending at the time of triggering and yet, the expression of these receptors is altered by culture conditions.^{54,56,57} What collectively understood from these studies is that the immunological state of MSCs is influenced predominantly by their environmental conditions. Hence, it is more likely that the conflicting reports about the immunoproperties of MSCs are due to different experimental and culture settings.

Although the underlying mechanisms are not still understood, studies have already shown that the immunosuppressive effect of MSCs may be exerted by the cell-to-cell interactions (HLA-G⁵⁸, PD-L1⁵⁹) and either by soluble factors secreted by MSCs or lymphocyte-affected MSCs.¹⁻³ Therefore, the physical separation of lymphocytes and MSCs, eliminating the cell-to-cell dependent mechanisms and diluting the reached inhibitory factors to lymphocytes (because of the distance between MSCs and lymphocytes) particularly about the unstable factors such as NO^{52,61} reduce the suppression and this have been shown about MSCs from different sources.^{33,34,45,62} On the contrary, some investigators reported that lymphocyte proliferation was not affected by the physical separation of the two populations.^{63,64} These studies point out that in the MSC-mediated immunosuppression; soluble factors play the key role and cell to cell mechanisms are dispensable. Since these results were obtained from different investigations which worked on a subset of immune cells and used different methods and sometimes different MSCs, it is possible that in such situation some mechanisms may become dominant. However, our results showed more inhibition of T cell proliferation in usual cocultures (direct contact) than transwell cocultures for both types of MSCs, but this difference was statistically significant only about BM-MSCs. If similar results are repeated in further complementary studies, this will mean that the cell-to-cell interactions in immunosuppression of SHED are not as important as BM-MSCs.

In the present work, by the reduction of the number of both MSCs (BM-MSCs and SHED) present in contact to lymphocytes, the inhibition of activated T cells diminished. This meant that immunosuppression of BM-MSCs and SHED was dose-dependent as it has been reported by other researchers about various MSCs from different tissue sources.^{44,62,65} We found a

significant proliferation inhibition even at MSC to T cell ratio of 1/100 for BM-MSCs and SHED; there is not another report about SHED, but this ratio for BM-MSCs differs noticeably in diverse studies.^{62,66,67} This diversity was probably due to various experimental settings.

Additionally, the detected inhibition of BM-MSCs was remarkably more than SHED on the whole and this superiority was maintained when the kind of stimulation, type of contact or the ratio of MSCs to lymphocytes were concerned individually. Since the conditions of cocultures were the same for both SHED and BM-MSCs, it can be concluded that BM-MSCs more efficiently suppressed the proliferation of activated T lymphocytes. This finding certainly should be confirmed by further studies.

The sequential events of T cell activation is cellular proliferation and secretion of cytokines, so we also determined the amount of some cytokines in the supernatant of cocultures by ELISA. The assayed cytokines were IL-2 and IFN- γ , which are important cytokines secreted chiefly from many activated T cells.⁶⁸ Moreover, MSCs seem not to produce these cytokines; nevertheless the cytokines produced by MSCs is still rudimentary.⁶⁹

As expected and also has been reported by numerous scientists,^{33,45} by the reduction of T cell proliferation in the presence of MSCs, the amount of assayed IL-2 and IFN- γ were reduced. This was observable about both cytokines in MLCs as well as LTTs cocultured with diminished number of BM-MSCs and SHED. By the comparison of IL-2 and IFN- γ production of T cells in direct and indirect contact to MSCs, the similar results were obtained, except about IFN- γ for BM-MSCs. In this case, despite detected higher inhibition in usual cocultures (direct contact), the amount of assayed IFN- γ (not IL-2) in these cocultures was slightly less than control cultures (culture of activated T cells without MSCs), while was significantly more than that of transwell cocultures (with higher proliferation). To explain this discrepancy, we should consider the following points:

In spite of the existence of a large body of studies which have explored the role of physical contact (of MSCs and lymphocytes) in immunoregulatory role of MSCs, and although in many of these studies beside the proliferation, the amount of IFN- γ and other cytokines (secreted by immune cells) were measured,^{18,22,70-72} they only displayed the decreased proliferation in

Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth

physical contact but did not pay attention to the probable changing cytokines in direct and indirect contacts. However Yañeza et al.⁷³ reported that the produced IFN- γ from activated DCs in indirect contact to MSCs was more than direct contact. In contrast, Krampera's work⁴¹ implied that no proliferation and no cytokine production from T CD4 cells was affected by cell contact. Noteworthy, Beyth⁶² observed that the reduction of secreted IFN- γ from T lymphocytes was dependent on the presence of monocytes in cell culture and finally Glennie et al.⁷⁴ showed that the proliferation of lymphocytes and their cytokine production may be affected in different ways by MSCs.

Concluded point from these stated investigations is that the suppression of IFN- γ production from lymphocytes and the inhibition of lymphocyte proliferation by MSCs was not necessarily the same, and might depend on culture conditions. So it is conceivable that in our investigational conditions, BM-MSCs diminished the proliferation and IFN- γ secretion of human T cells in different ways.

As mentioned above, the amount of assayed IL-2 was more compatible to T cell proliferation that was reasonable. IL-2 is a cytokine that is produced from activated T lymphocytes,⁶⁸ thus more IL-2 was expected when there existed higher number of activated T cells (more proliferation).

The results of this study provided evidence for SHED, as expected from various tissue-derived MSCs, that could suppress the activation of human T lymphocytes in vitro. However, compared to BM-MSCs, this suppression, at least in our experimental condition was distinctly alleviated. Moreover, it seemed that SHED did not exert the exact mechanisms of BM-MSCs' immunosuppression, given that in the equal condition, the pattern of immune-modulation of BM-MSCs and SHED was different. This may be well justified by investigations which have established that MSC from different tissue origins share many features but are not identical.⁷⁵ This finding certainly should be verified by further studies directed to identify the detailed mechanisms responsible for the immunomodulation of SHED and also BM-MSCs.

In conclusion, stem cells from human exfoliated deciduous teeth (SHED) with similar phenotyping; differentiation and proteomic characteristics to MSCs had also immune properties of MSCs. This plentiful and readily accessible cell population can be a suitable alternative source of MSCs rather than bone marrow

for experimental, clinical and preclinical works.

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Immunoregulatory Effects of Stem Cells from Human Exfoliated Deciduous Teeth

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