Effect of Mesenchymal Stem Cells on ILT3 Expression in the Splenocytes of Skin Graft Recipient Mice

Ali Moravej[1,2], Mohammad-Hossein Karimi[1]*, Bita Geramizadeh[1], Mahdokht Hossein Aghdaie[1], Omid Kohi-Hoseinabadi[3], Salimeh Ebrahimnezhad[1]

[1]Transplant Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, [2]Noncommunicable Diseases Research Centre, Fasa University of Medical Sciences, Fasa, [3]Laboratory Animals Center, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Mesenchymal stem cells (MSCs) are considered as effective therapeutic cells in transplantation due to their immunomodulatory activities. However, precise mechanism of MSCs immunomodulatory activity is not completely understood.
Objectives: To study the role of Immunoglobulin-like transcripts-3 (ILT3) immunomodulatory receptor in immune tolerance induced by MSCs in skin transplantation model and induction of tolerogenic dendritic cells (Tol-DCs) by MSCs through up-regulation of ILT3. **Methods:** C57BL/6 skin grafts were transplanted to the back of BALB/c mice. Recipient mice received MSCs on days 0, 1 and 2 post transplantation. On days 2, 5 and 10 post skin transplantation, ILT3 and forkhead box P3 (FOXP3) expression in the spleens of MSCs treated mice were evaluated. Furthermore, MSCs and DCs were co-cultured in cell culture plates and transwell systems. Then, the expressions of ILT3 mRNA and protein in MSC-treated DCs were evaluated. Additionally, MSC-treated DCs were co-cultured with allogeneic T-cells and FOXP3 expression in T-cells was evaluated. **Results:** The expression of ILT3 and FOXP3 were higher in the splenocytes of MSCs-treated mice early post-transplantation. Furthermore, we observed that MSC-treated DCs can increase FOXP3 expression in T-cells. But, we could not find any differences in ILT3 expression between MSC-treated DCs and untreated ones. **Conclusion:** One of the mechanisms underlying MSCs immunomodulatory function could be up-regulating ILT3 expression in splenocytes. But our results did not support the hypothesis that MSCs induce Tolergenic DCs by up-regulation of ILT3.

Moravej A, et al. Iran J Immunol. 2016; 13(3):274-288.

Keywords: ILT3, Immunomodulation; MSCs, Transplantation

---

*Corresponding author: Dr. Mohammad-Hossein Karimi, Transplant Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran, Tel: (+) 98 917 3149022, Fax: (+)98 711 6474331, e-mail: Karimimh@sums.ac.ir

INTRODUCTION

Today, organ transplantation is one of the most effective therapeutic methods used for patients with organ failures. However, graft rejection is still the major problem in allogeneic transplantations. Recently, mesenchymal stem cells (MSCs) are considered as effective therapeutic cells in transplantation due to their immunomodulatory activities (1-6). These multi-potent stem cells have self-renewal ability and are capable of migrating and engrafting at the sites of inflammation (7). Furthermore, MSCs modulate most cells of the innate and adaptive immunities such as B-lymphocytes, T-lymphocyte, Natural Killer (NK) cells and antigen-presenting cells (APCs) (7-9) by direct contact or by secretion of different chemokines, cytokines and growth factors (7). In this regard, it has been shown that MSCs can inhibit T-lymphocytes activation and proliferation and induce generation of regulatory T-cells (Treg) (8,9). Also, it is reported that MSCs are able to alter immunogenic dendritic cells (DCs) to the tolerogenic ones (10).

DCs are conventionally known to be able to activate T-cells. However, some studies revealed that DCs are also able to exhibit tolerogenic activities such as differentiation of T-cells to Treg cells (11). Bidirectional interactions between DCs and T-lymphocytes determine either activation of immunogenic or tolerogenic immune response which plays a crucial role in the outcome of malignancies, autoimmune diseases and transplantations. In contrast with immunogenic DCs, the tolerogenic DCs (Tol-DCs) express low levels of co-stimulatory molecules as well as high levels of immunomodulatory receptors (11). The examples of such immunomodulatory receptors expressed by Tol-DCs are Immunoglobulin-like transcripts (ILTs), also known as leukocyte immunoglobulin-like receptors (LIRs), which can inhibit the activation of both DCs and T cells (12-14).

ILTs share considerable homology with natural killer cell Ig-like receptors (KIRs). Some of the ILTs such as ILT2, ILT3, ILT4 and ILT5 have a long cytoplasmic tail that contains several immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and similar to inhibitory NK cell receptors have inhibitory functions and prevent cellular activation (15). On the contrary, some of them like ILT1, ILT7 and ILT8 have a much shorter cytoplasmic tail similar to the activating NK cell receptors which contain immunoreceptor tyrosine-based activation motifs (ITAM) (15). There are several studies showing immunoregulatory roles of ILTs expressed on some of the myeloid cells such as mast cells, neutrophils, macrophages, NK cells and activated T-lymphocytes as well as APCs (15). DCs expressing high level of ILT3 display lower phosphorylation of NF-κB and decreased capacity of transcription of NF-κB dependent co-stimulatory molecules and are unable to stimulate the T-helper proliferation and instead differentiate them to Treg cells (11,14,16). In addition, ILT3 knockdown DCs secrete high levels of cytokines and chemokines leading to strong T-lymphocyte responses to alloantigens(17). As reported, tolerogenic DCs direct the generation of Treg cells from naive T-cells, and conversely, Treg cells induce the differentiation of immature DCs into Tol-DCs. Meanwhile, Treg cells increase the expression of ILT3 in DCs and decrease the expression of co-stimulatory molecules leading to the induction of tolerance (14,17). After activation and down-regulation of ILT3 gene, DCs lose their tolerogenic function and become immunogenic (13). Previous studies demonstrated that the expression of ILT3 on DCs is strongly regulated by different inflammatory stimuli, growth factors, immunosuppressive factors and cytokines such as IL-10 (11,13).

Further studies in the mechanism of MSCs immunomodulatory activity could be useful for the development of clinical application of these cells in transplantation. Given the significance of ILT3 as immunomodulatory receptor, we decided to investigate the role of ILT3 in immune tolerance acquired by bone marrow derived MSCs (BM-MSCs) in skin transplantation mouse model. Furthermore, we hypothesized that this molecule could be involved in the induction of Tol-DCs by MSCs. In doing so, we investigated the effect of MSCs on the expression of ILT3 by DCs.

MATERIALS AND METHODS

Isolation and Characterization of mice MSCs. MSCs were isolated from bone marrow of femurs and tibiae of 8-12 week old BALB/c (H-2Kd) mice (18). Four passages were done prior to use of the cells to achieve the highest purity. The purity of bone marrow derived MSCs (BM-MSCs) was assessed by Flowcytometry using anti-CD44, Sca-1, CD34 and CD45 surface markers.

Skin Transplantation and MSCs Administration.8–12 week old BALB/c (H-2Kd) and C57BL/6 (H-2Kb) mice were purchased from central animal laboratory, Shiraz University of Medical Sciences, Iran. Ketamine/Xylazine cocktail was used as an anesthetic agent. 1.5 cm^2 full-thickness skin grafts were obtained from the back-skin of C57BL/6 mice and transplanted to the back of BALB/c mice, sutured with six stitches and secured with Vaseline gauze and a bandage. Bandages were removed seven days after transplantation and grafts that failed up before day 7 post transplantation were considered as technical error and excluded from the study. Recipient mice received MSCs (intraperitoneal injection, 5 x 10^5 Cells) exactly after completion of the skin transplantation procedure, and also on days 1 and 2 post transplantation (intraperitoneal injection, 1 x 10^6 Cells). The maintenance and care of experimental animals complies with National Institute of Health guidelines for the humane use of laboratory animals. Mice were sacrificed by cervical dislocation. All of the surgeries were done under the supervision of institutional animal care and use committee and also animal ethics committee of Shiraz University of Medical Sciences.

In-Vivo Study Groups. Totally, 6 groups were defined in this study: Groups 1-3 included the mice that received allogeneic skin transplantation and PBS injection; they were sacrificed on days 2, 5 and 10 post transplantation, respectively. The mice of groups 4-6 received allogeneic skin transplantation and intraperitoneal infusion of MSCs and were sacrificed on days 2, 5 and 10 post transplantation, respectively. Each group included at least 5 mice, and 3 independent experiments were performed. (Table 1).

Evaluation of ILT3 and FOXP3 mRNA in the Splenocytes of Recipient Mice by Realtime PCR. On days 2, 5 and 10 post skin transplantation, the spleens of mice were harvested and became single cell by mechanical dissociation with sterile mesh. Red blood cells were lysed and the remained cells were recovered with NycoPrep density gradient medium (Axis-shield, Norway). Cells were collected from the NycoPrep interface and the levels of ILT3 and FOXP3 mRNA expression were evaluated by Real-time PCR. Briefly, total RNA was extracted by TRIzol reagent (Invitrogen, USA) and cDNA was synthesized by cDNA synthesis kit (Takara, Japan), according to manufacturer's protocol. ILT3 specific primers (forward: 5′-GGCTACTTTAGAAATGAACCACAG -3′ and reverse: 5′-

AGGAGGAATGACACCAGGAC -3′), FOXP3 specific primers (forward: 5′-AATAGTTCCTTCCCAGAGTTCTTC-3′ and reverse: 5′-ATGGTAGATTTCATTGAGTGTCCT-3′) and GAPDH specific primers as internal control (forward: 5′- CGGTGTGAACGGATTTGGC -3′ and reverse 5′-GTGAGTGGAGTCATACTGGAAC -3′) were designed by AlleleID software (Version 7.5). Sybr Green Realtime PCR was performed using Realtime PCR System (ABI step one plus, Applied Biosystems, USA). Finally, fold change of expression was calculated using the equation $2^{-\Delta\Delta Ct}$. Each group included at least 5 mice, and 3 independent experiments were performed.

Table 1. Groups defined for studying the effects of MSCs on ILT3 expresion.

Groups	Donor	Recipient	MSC Intraperitoneal Injection	Sacrifice day
1	BALB/c	C57BL/6	No	2
2	BALB/c	C57BL/6	No	5
3	BALB/c	C57BL/6	No	10
4	BALB/c	C57BL/6	Yes	2
5	BALB/c	C57BL/6	Yes	5
6	BALB/c	C57BL/6	Yes	10

Evaluation of ILT3 Protein Expression in the Splenocytes of Recipient Mice by WesternBlot. RIPA buffer (Sigma, USA) was used for the extraction of proteins from Splenocytes. Cell extracted proteins were separated on 12% SDS polyacrylamide resolving gel with a 3% stacking gel and transferred to the polyvinylidene fluoride (PVDF) membrane (GE healthcare, USA). Armenian hamster anti-mouse ILT3 (0.5 µg/ml) (Biolegend, USA) and HRP-conjugated rabbit anti-Armenian hamster antibody (1:2000 dilution) (Abcam, USA) were used to detect ILT3. HRP-conjugated rabbit anti-mouse β-actin (1:4000 dilution) (Abcam, USA) was used for the detection of β-actin, as internal control. Electrochemiluminescence (ECL) (GE Healthcare, USA) was used as substrate and the resulting signal was visualized using ChemiDoc imaging system (Bio-Rad, USA). The intensities of the protein bands were quantified using GelAnalyzer software (Version 2010). The intensities of the ILT-3 protein bands were normalized with the intensity of β-actin bands in each sample.

Dendritic and T-Cells Isolation. DCs were isolated from the spleens of BALB/c mice. Briefly, mice spleens were chopped and digested with 1 mg/ml collagenase D (Roche, Germany), then meshed with 0.2 µm sieve. The cell suspension was layered on Nycodenz solution. The ring was collected and purified using a positive selection magnetic adsorption cell sorting (MACS) technique by CD11c Micro Beads kit (Miltenyi Biotechnology, Germany), according to the manufacturer's instructions. $CD4^+$T-cells were isolated from draining lymph nodes of C57BL/6 mice using a negative selection MACS technique by $CD4^+$ T-cell isolation Micro Beads kit (Miltenyi

Biotechnology, Germany). Purity of the isolated DCs and T-cells was respectively assayed by specific PE-conjugated anti CD11c antibody and specific PE-conjugated anti-CD4 antibody using flowcytometry.

In-Vitro Study Groups. Six-well cell culture plates and transwell systems (0.8 μm pore size membrane, SPL, USA) were used for MSCs/DCs co-culture. Cells were cultured in RPMI medium, supplemented with 1% penicillin/streptomycin and 10% FBS and 50 ng/mL GM-CSF (Peprotech, USA). In one of each pair of wells, LPS (200 ng/mL) (Sigma, USA) was added to induce DCs maturation. In transwell systems, MSCs and DCs were respectively placed in the upper and lower chambers of the transwell plates at 1:10 and 1:50 ratios. Furthermore, according to table 2, in some conditions, DCs were directly seeded onto MSCs at the same ratios. Each well contained 2 x 10^6 DCs. Cells were co-cultured for 24 hours. Then, DCs were collected to study the gene expressions and also mixed leukocyte reaction (MLR). Untreated DCs were used as negative control in the presence or absence of LPS. Each group included 5 experiment was, and each experiment repeated 3 times, independently (Table 2).

Table 2. Groups designated for study the effects of MSCs on ILT3 expression in DCs

Groups	MSCs/DC ratio	Co-culture/ Transwell	LPS
1	(1:10)	Co-culture	-
2	(1:10)	Co-culture	+
3	(1:10)	Transwell	-
4	(1:10)	Transwell	+
5	(1:50)	Co-culture	-
6	(1:50)	Co-culture	+
7	(1:50)	Transwell	-
8	(1:50)	Transwell	+
9	DCs only	-----	-
10	DCs only	-----	+

Evaluation of ILT3 mRNA and Protein Expression in DCs Treated with MSCs. MSCs treated DCs were collected to study the expressions of ILT3 mRNA and protein by real time-PCR and Western blot, as previously described.

Mixed Leukocyte Reaction (MLR) and FOXP3 mRNA Expression Evaluation.MSCs treated DCs were inactivated with irradiation (30 Gy). Then, cells were washed with PBS for three times and resuspended in complete culture medium. MLR was set up in 96- well round-bottom cell culture plates (Nunc, Denmark). 1×10^4

cells of MSCs treated DCs (BALB/c), as stimulator cells, were co-cultured with 1×10^5 cells of allogeneic T-cells (C57BL/6), as responder cells in a total volume of 200 µl. After 48 hours of incubation, FOXP3 mRNA expression in T-cells were evaluated. Negative control was allogeneic T-cells co-cultured with untreated DCs.

Statistical Analysis.SPSS software version 16 was used to analyze data. Kruskal–Wallis and Mann–Whitney tests were used to compare differences in mRNA and protein expression among the study groups. The results were expressed as mean ± SD. Level of significance was set at less than 0.05. Correlations were analyzed using Spearman test.

RESULTS

FlowcytometricAnalysis. The purity of MSCs was assessed by phycoerythrin (PE) conjugated anti Sca-1, anti-CD44, anti-CD45 and fluorescein isothiocyanate(FITC) conjugated anti-CD34 antibodies as well as proper isotype controls (all from eBioscience, USA). Flowcytometric analysis showed that about 95% of cells were positive for CD44 and Sca-1 and negative for CD34 and CD45 markers. Purities of the isolated DCs and T-cells were respectively assayed by specific PE-conjugated anti-CD11c antibody and specific PE-conjugated anti CD4 antibody using flowcytometry. The purities of DCs and T-cells were found to be more than 90% (Figure 1).

Evaluation of ILT3 and FOXP3 Expressions in the Splenocytes of Skin Graft Recipient Mice.To study the expressions of ILT3 and FOXP3, Real-time PCR and Western blot were performed on the splenocytes of skin graft recipient mice. ILT3 mRNA expression in MSCs treated mice was higher, in comparison to control mice on day 2 and 5 post transplantation, although the latter was not statistically significant (P = 0.007 and p = 0.14, respectively). The results of ILT3 protein expression confirmed the ILT3 mRNA results. A protein with a molecular weight of about 49 kDa related to ILT3 was detected in the spleens of all groups. ILT3 protein expression in MSCs treated mice was higher than in the controls on days 2 and 5 post transplantation (p=0.043 and p=0.035, respectively) (Figure 2).

Furthermore, FOXP3 mRNA expression in MSCs treated mice was statistically higher, compared to control mice on days 2, 5 and 10 post transplantation (P=0.006, P=0.004 and p=0.023, respectively) (Figure 2).

Correlation between ILT3 and FOXP3 Expressions in the Splenocytes of Skin GraftRecipientMice. There was a significant positive correlation between ILT3 mRNA and protein expressions (p=0.015). Furthermore, we found a positive correlation between FOXP3 mRNA expression and ILT3 mRNA and protein expression (p=0.002 and p=0.034, respectively).

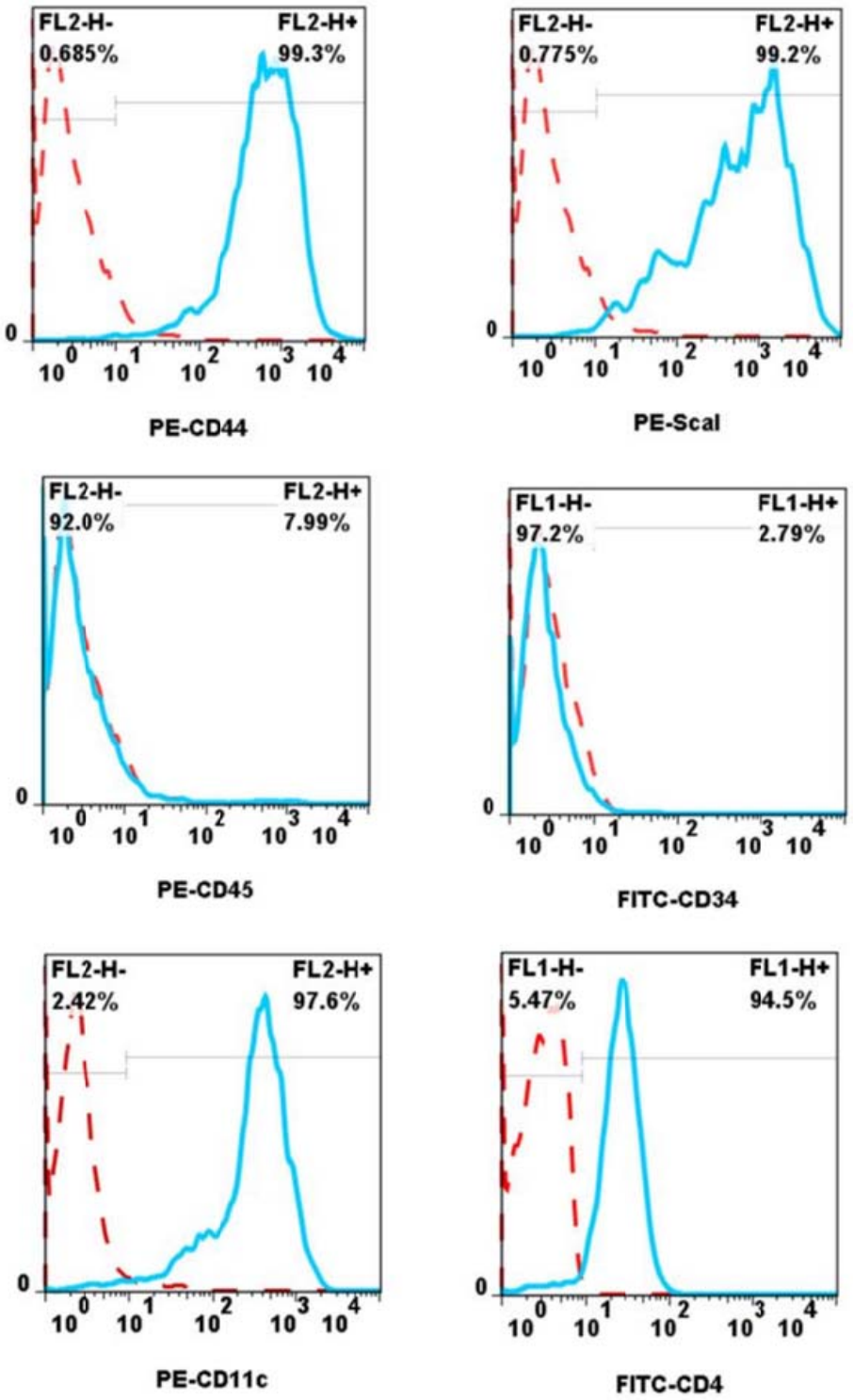

Figure 1.Purity of isolated MSCs, DCs and T-cells. Purity of isolated MSCs was evaluated using antibodies against MSCs markers (CD44 and Sca-1) and antibodies against hematopoietic cell lineages (CD34 and CD45) by flow cytometry. The results showed that the purity of isolated MSCs was more than 95%. Purity of DCs and T-cells were respectively evaluated by anti-CD11c and CD4 antibodies. The purity of these cells was more than 90%. (Specific antibodies: solid histograms, isotype control antibodies: dashed histograms)

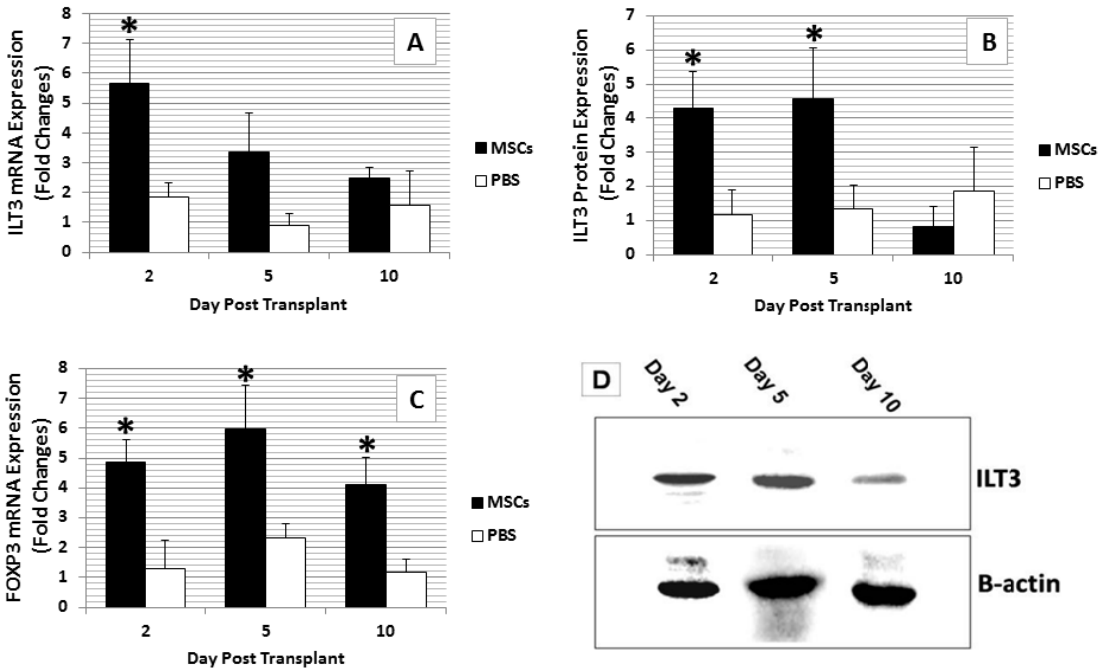

Figure 2.ILT3 and FOXP3 expressions in spleens of the mice receiving skin allograft. On days 2, 5 and 10 post skin transplantation, ILT3 and FOXP3 mRNA expressions were measured in splenocytes of mice using RT-qPCR (A and B). Furthermore, ILT3 protein expressions in the study groups were measured using Western blot. (C). Each group included at least 5 mice. Data represent the mean ± SD of 3 independent experiments. Proteins with molecular weights of about 42 kDa and 49 kDa related to β-actin and ILT3 were detected in the spleens of all groups (D).

Effect of MSCs on ILT3 mRNA and Protein Expression in DCs Treated with MSCs. ILT3 mRNA and protein expressions in DCs treated with MSCs were evaluated by Real-time PCR and Western blotting. There were no statistically significant differences in the expressions of ILT3 mRNA and protein between DCs co-cultured with MSCs, DCs treated with MSCs in transwell and untreated ones (all $p>0.05$), although an increase in ILT3 mRNA expression in DCs co-cultured with MSCs in 1:10 ratio was observed (Figures 3 and 4).

Effects of MSCs-Treated DCs on FOXP3 mRNA Expression in MLR. The results showed that in the absence of LPS, FOXP3 expression was higher in MLRs containing DCs co-cultured with MSCs in 1:10 and 1:50 ratios, in comparison with untreated DCs ($p<0.001$). Similarly, in the presence of LPS, FOXP3 expression was higher in MLRs containing DCs co-cultured with MSCs in 1:10 and 1:50 ratios, compared to untreated DCs ($p<0.001$). However, there was no statistically significant difference in FOXP3 expression in MLRs containing DCs treated with MSCs in transwell and untreated DCs in the presence or absence of LPS (all $p>0.05$) (Figures 3 and 4).

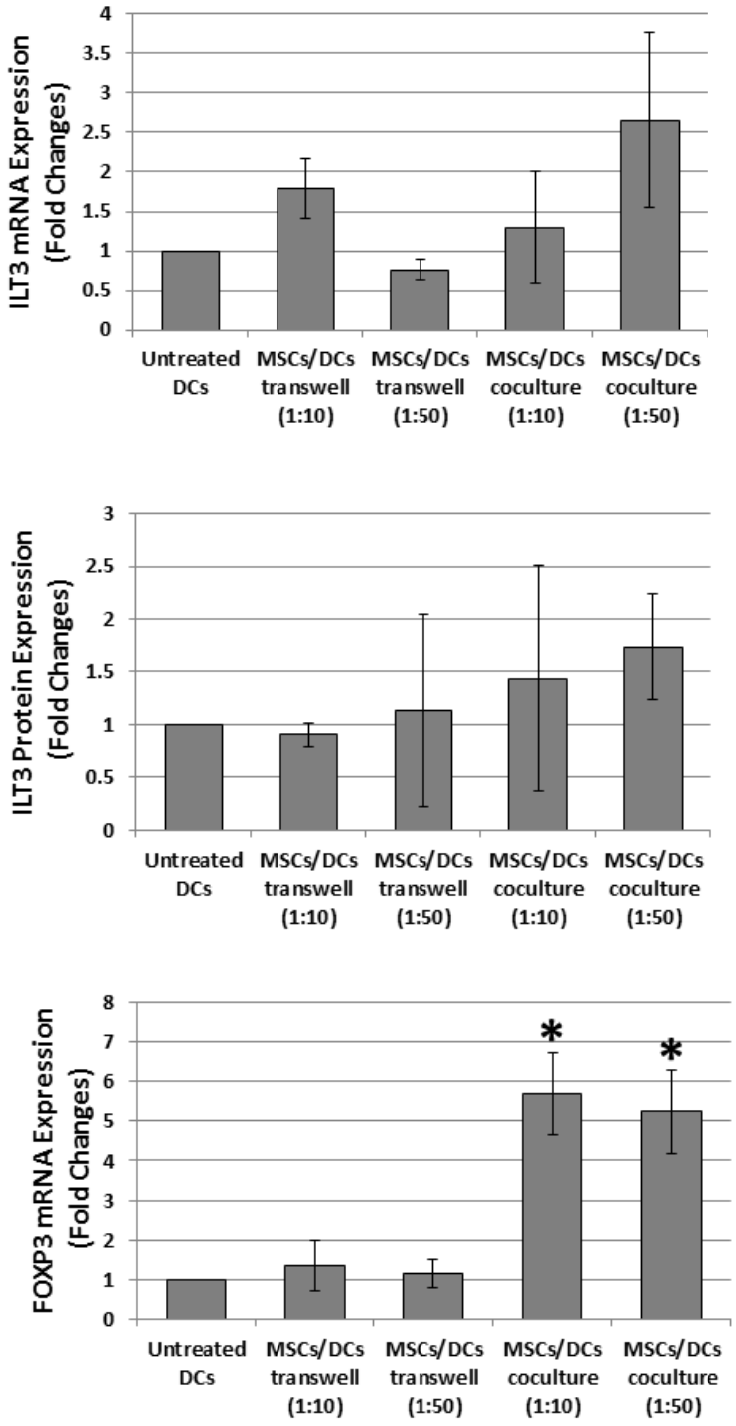

Figure 3. ILT3 and FOXP3 expressions in DCs treated with MSCs and T-cells in the presence of LPS. mRNA expressions of ILT3 and FOXP3 were measured in MSCs treated DCs and T-cells in the presence of LPS (A and B). Furthermore, protein expressions of ILT3 MSCs treated DCs were measured using Western blot (C). Data represent the mean ± SD of 5 experiments in each group.

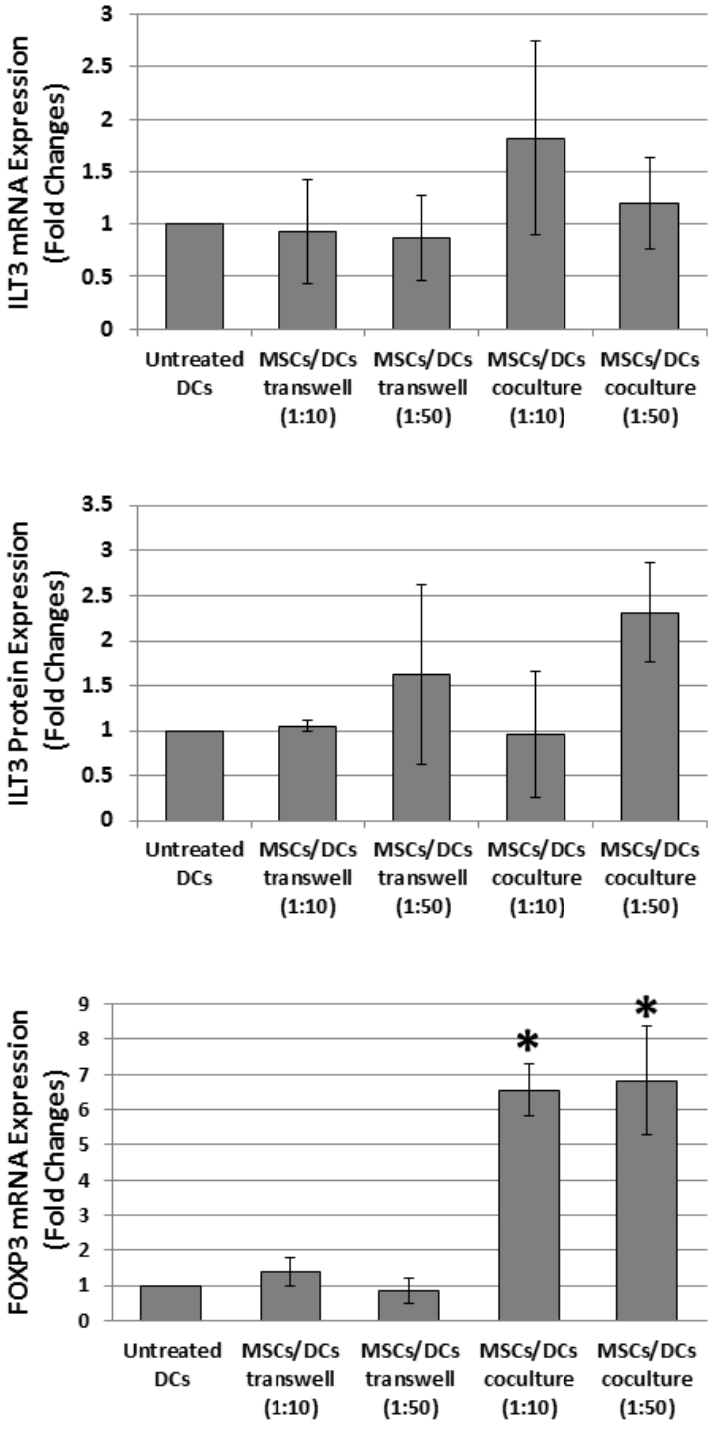

Figure 4. ILT3 and FOXP3 expressions in MSCs treated DCs and T-cells in the absence of LPS. mRNA expressions of ILT3 and FOXP3 were measured in MSCs treated DCs and T-cells in the absence of LPS (A and B). Furthermore, protein expressions of ILT3 MSCs treated DCs were measured using western blot (C). Data represent the mean ± SD of 5 experiments in each group.

DISCUSSION

Previous studies revealed that the administration of MSCs could prolong the solid organ graft survival time in humans and mice (19-21). In this regard, in our previous study, we found that MSCs administration can prolong skin graft survival time from 11 days in untreated mice to 14 days in mice received MSCs (22). But the precise mechanism of MSCs immunomodulatory function is not completely known. Herein, we hypothesized that treatment with MSCs can enhance the expression of ILT3 tolerogenic receptor in the splenocytes of the mice receiving skin allografts. Accordingly, we evaluated and compared the expression of ILT3 at the mRNA and protein levels in the splenocytes of MSCs treated and control mice on days 2, 5 and 10 post skin allogeneic transplantation. Our results demonstrated that MSCs administration increases the expression of ILT3 at both mRNA and protein levels in the splenocytes of allogeneic skin graft recipient mice. So, we deduced that MSCs could suppress immune system via up-regulation of ILT3 and consequently result in prolongation of graft survival time. Surprisingly, at the beginning days post transplantation, expression of this molecule in MSCs treated mice was high, but over the time its expression decreased which could be because injected MSCs were eliminated or died or lost their imunoregulatory capability (19,23).

The key feature that makes MSCs therapeutic cell candidates for allograft transplantations is the diversity of their immunomodulatory activities affecting adaptive immune responses, particularly by differentiating T-cells to Treg cells (1,24). In agreement with the previous studies, our results showed that FOXP3 in the splenocytes of MSCs treated mice was higher in comparison to the controls on days 2, 5 and 10 post transplantation. Moreover, down-regulation of FOXP3 in MSCs-treated mice was concomitant with decreased expression of ILT3.

It was reported that MSCs are able to alter immunogenic dendritic cells (DCs) to tolerogenic ones that cannot activate T-cells, but differentiate them to Treg cells (10). Moreover, reports indicate that DCs expressing a high level of ILT3 show lower phosphorylation of NF-κB and decreased expression of co-stimulatory molecules and these DCs are unable to stimulate T-helper proliferation and instead differentiate them to Treg cells (11,14,16,25-27). Taken together, we hypothesized that one of the mechanisms involved in the induction of Tol-DCs by MSCs could be the up-regulation of ILT3 in DCs. To investigate this, DCs were treated with MSCs and expression of ILT3 on DCs was evaluated. Besides, we aimed to investigate whether immunomodulatory effect of MSCs on DCs is dependent on cell to cell contact or is just mediated by soluble factors. In doing so, DCs were directly co-cultured with MSCs and also treated with MSCs in a transwell plate system. Additionally, the capability of treated DCs to generate Treg cells in MLR was evaluated. The results of our study showed that DCs co-cultured with MSCs and also DCs treated with MSCs in transwell plates were able to increase the expression of FOXP3 which is the main marker of Treg cells. The results of our study revealed very low level of ILT3 mRNA and protein expression in MSCs-treated DCs as well as untreated ones. Moreover, we could not find any differences in ILT3 mRNA and protein expression between MSCs treated DCs and untreated ones. These data refute the hypothesis that MSCs induce Tol-DCs by up-regulation of ILT3. There are a few studies investigating the effect of MSCs on the expression of ILT3 on DCs. In this regard, Dokic *et al*. showed that periapical lesions derived mesenchymal stem cells (PL-MSCs) can prevent differentiation of monocyte to DCs via soluble factors. Furthermore, they found that DCs which were cultivated with

PL-MSCs and pro-inflammatory cytokines, although showed mature phenotype, induced T-cell anergy, had a weak allostimulatory function, polarized T-cells to Th2, differentiated $CD4^+$ T-cells to Treg cells and increased the secretion of TGF-β cytokine via IDO, ILT-3 and ILT-4 dependent mechanisms. Also, they revealed that DCs which were co-cultured with PL-MSCs expressed 64 to 87 folds more ILT-3, in comparison with untreated DCs (28). These observations were different from ours, because we did not find any difference in the expression of ILT-3 in BM-MSCs treated DCs and untreated ones. It is worth mentioning that Dokic *et al.* co-cultured MSCs and DCs for 6 days while in our study, the cells were co-cultured for 24 hours, which could be a limitation of our study. Perhaps more than 24 hour stimulation of DCs by MSCs is needed to increase the expression of ILT3 on DCs. Furthermore, we used mouse cells, while they studied on human cells. Another reason of the difference between our results and Dokic *et al.* observation could be due to using varying sources of the MSCs and DCs. In our study, we used bone marrow derived MSCs and spleen derived DCs, while they used periapical lesions derived mesenchymal stem cells and monocyte derived DCs.

In another study by Wehner *et al.* the effect of MSCs on the pro-inflammatory capacity of 6-sulfo LacNAc (slan) DCs, the main subpopulation of human blood DCs, was investigated. They revealed that BM-MSCs can considerably inhibit the maturation of slanDCs and their capability to secrete pro-inflammatory cytokines. Moreover, they demonstrated that MSCs can increase the expression of ILT3 and ILT4 on slanDCs(29). The reason of the difference between our results and Wehner*et al.* observations could be explained by the difference between the sources of the DCs and T-cells. In our study, we used mouse splenic DCs and T-cells derived from lymph node, while they used PBMC derived DCs and T-cells. Furthermore, we used mouse cells, while they studied on human cells. Additionally, the techniques used to detect ILT-3 were different in Wehner*et al.* study and ours, that is, they used flowcytometery technique, while we used Realtime PCR and Western Blot techniques.

In a study carried out by Hancharou *et al*., human olfactory mucosa-derived MSCs (hOM-MSCs) were co-cultured with DCs and investigated the effects of these cells on the expression of some immunogenic and tolerogenic markers on DCs. They found that hOM-MSCs significantly increased the expression of both immunogenic (CD86) and tolerogenic markers (CD85k) of DCs. The reason of the difference between our results and Hancharou *et al.* observations could be due to using different sources of the DCs and MSCs. In our study, we used mouse splenic DCs and MSCs derived from mouse bone marrow, while they used hOM-MSCs and DCs which were differentiated from human monocytes using standard 6-day (GM-CSF/IL-4) protocol (30).

In some studies, it was reported that the expression of ILT3 at both mRNA and protein levels in DCs is decreased after activation by inflammatory stimuli (13). For example, Ju*et al*. studied the ILTs expression in human PDCs, Mo-DCs and DCs differentiated from $CD34^+$ progenitor cells and showed that CpG-DNA and inflammatory stimuli such as TNF-α and soluble CD40 ligand down-regulate the expression of ILT2 and ILT3 at both mRNA and protein levels (13). In the present study, we aimed to show if the alteration of DCs to TolDCs which probably express higher levels of ILT3 under the influence of MSCs is reversible in the presence of maturation inducing molecules like LPS or not. For this purpose, we used conditions with LPS. The results revealed no difference between the expression of ILT3 in DCs in the presence and absence of LPS. The difference between our results and those of Ju *et al.* could be explained by

difference in the source of DCs and also difference of stimulators. In another study carried out by Kasai *et al.,* the surface expression of ILT3 on the splenic and bone marrow derived DCs (BM-DCs) of mice and also on the DCs after stimulation with LPS was examined by flowcytometry. They found that ILT3 is considerably expressed on bone marrow derived DCs, but similar to our results they found low expression of this molecule on splenic DCs. Moreover, in agreement with our results, they found no change in ILT3 expression even after 24 hours stimulation of BM-DCs and splenic DCs by LPS (31).

We found that BM-MSCs can increase the expression of ILT3 immunomodulatory receptor in both mRNA and protein levels in splenocytes, which may cause the prolongation of skin allograft survival time. Furthermore, according to our results, prolonged transplant survival in BM-MSCs treated mice seems to be associated with higher expression of FOXP3 in the spleen. Our data suggest that ILT3 expression in the splenocytes of the recipient mice treated with MSCs, can down-regulate their alloreactive T-lymphocytes responses and up-regulate Treg generation, thus, limits the immune responses leading to increased graft survival. Also, the results showed that in in-vitro, DCs co-cultured with MSCs and also DCs treated with MSCs in transwell plates, were able to increase the expression of FOXP3 which is the main marker of Treg cells in MLR. However, we could not find any differences in ILT3 mRNA and protein expression between MSCs treated DCs and untreated ones. These data do not support the hypothesis that MSCs induce Tol-DCs by up-regulation of ILT3 in DCs.

ACKNOWLEDGEMENTS

This study was a part of PhD thesis written by Ali Moravej, and financially supported by the grant No. 92-6771 awarded by Shiraz University of Medical Sciences and grant No. 92/195 by Transplant Research Center. Also, our thanks are due to Dr. Hassan Khajehei for linguistic copy editing of the manuscript.

REFERENCES

1. Landgraf K, Brunauer R, Lepperdinger G, Grubeck-Loebenstein B. The suppressive effect of mesenchymal stromal cells on T cell proliferation is conserved in old age. Transpl Immunol. 2011; 25:167-72.
2. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008; 371:1579-86.
3. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004; 363:1439-41.
4. Karimi MH, Marzban S, Hajiyan MR, Geramizadeh B, Pourfathollah AA, Rajabiyan MH, et al. Effect of CD40 silenced dendritic cells by RNA interference on mice skin allograft rejection. Immunotherapy. 2015; 7:111-8.
5. Langroudi L, Hassan ZM, Soleimani M, Hashemi SM. Tumor Associated Mesenchymal Stromal Cells Show Higher Immunosuppressive and Angiogenic Properties Compared to Adipose Derived MSCs. Iranian journal of immunology : IJI. 2015; 12:226-39.
6. Ahmadi-Ashtiani HR, Allameh A, Rastegar H, Mortaz E, Saraf Z. Immunoregulatory effects of glutathione during mesenchymal stem cell differentiation to hepatocyte-like cells. Iran J Immunol. 2012; 9:175-87.

7. Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, et al. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells. 2014; 6:552-70.
8. Yi T, Song SU. Immunomodulatory properties of mesenchymal stem cells and their therapeutic applications. Arch Pharm Res. 2012; 35:213-21.
9. Stagg J, Galipeau J. Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. Current molecular medicine. 2013; 13:856-67.
10. Sadeghi L, Kamali-Sarvestani E, Azarpira N, Shariati M, Karimi MH. Immunomodulatory effects of mice mesenchymal stem cells on maturation and activation of dendritic cells. Iran J Immunol. 2014; 11:177-88.
11. Manavalan JS, Rossi PC, Vlad G, Piazza F, Yarilina A, Cortesini R, et al. High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. Transpl Immunol. 2003; 11:245-58.
12. Wu J, Horuzsko A. Expression and function of immunoglobulin-like transcripts on tolerogenic dendritic cells. Hum Immunol. 2009; 70:353-6.
13. Ju XS, Hacker C, Scherer B, Redecke V, Berger T, Schuler G, et al. Immunoglobulin-like transcripts ILT2, ILT3 and ILT7 are expressed by human dendritic cells and down-regulated following activation. Gene. 2004; 331:159-64.
14. Suciu-Foca N, Cortesini R. Central role of ILT3 in the T suppressor cell cascade. Cell Immunol. 2007; 248:59-67.
15. Cella M, Dohring C, Samaridis J, Dessing M, Brockhaus M, Lanzavecchia A, et al. A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing. J Exp Med. 1997; 185:1743-51.
16. Li J, Liu Z, Jiang S, Cortesini R, Lederman S, Suciu-Foca N. T suppressor lymphocytes inhibit NF-kappa B-mediated transcription of CD86 gene in APC. J Immunol. 1999; 163:6386-92.
17. Chang CC, Ciubotariu R, Manavalan JS, Yuan J, Colovai AI, Piazza F, et al. Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nat Immunol. 2002; 3:237-43.
18. Mohtasebi MS, Nasri F, Kamali Sarvestani E. Effect of DiD Carbocyanine Dye Labeling on Immunoregulatory Function and Differentiation of Mice Mesenchymal Stem Cells. Stem Cells Int. 2014; 2014:457614.
19. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008; 20:55-60.
20. Sbano P, Cuccia A, Mazzanti B, Urbani S, Giusti B, Lapini I, et al. Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model. Arch Dermatol Res. 2008; 300:115-24.
21. Larocca RA, Moraes-Vieira PM, Bassi EJ, Semedo P, de Almeida DC, da Silva MB, et al. Adipose tissue-derived mesenchymal stem cells increase skin allograft survival and inhibit Th-17 immune response. PloS one. 2013; 8:e76396.
22. Moravej A, Karimi MH, Geramizadeh B, Azarpira N, Zarnani AH, Yaghobi R, et al. Mesenchymal Stem Cells Upregulate the Expression of PD-L1 But Not VDR in Dendritic Cells.Immunol Invest. 2016; 13:1-17.
23. Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood. 2005; 106:4057-65.
24. Prevosto C, Zancolli M, Canevali P, Zocchi MR, Poggi A. Generation of CD4+ or CD8+ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. Haematologica. 2007; 92:881-8.
25. Sanchez N, Miranda A, Funes JM, Hevia G, Perez R, de Leon J. Oncogenic transformation tunes the cross-talk between mesenchymal stem cells and T lymphocytes. Cell Immunol. 2014; 289:174-84.
26. Yang H, Sun J, Li Y, Duan WM, Bi J, Qu T. Human umbilical cord-derived mesenchymal stem cells suppress proliferation of PHA-activated lymphocytes in vitro by inducing CD4(+)CD25(high)CD45RA(+) regulatory T cell production and modulating cytokine secretion. Cell Immunol. 2016; 302:26-31.
27. Batorov EV, Shevela EY, Tikhonova MA, Batorova DS, Ushakova GY, Sizikova SA, et al. Mesenchymal stromal cells improve early lymphocyte recovery and T cell reconstitution after

autologous hematopoietic stem cell transplantation in patients with malignant lymphomas. Cell Immunol. 2015; 297:80-6.
28. Dokic J, Tomic S, Markovic M, Milosavljevic P, Colic M. Mesenchymal stem cells from periapical lesions modulate differentiation and functional properties of monocyte-derived dendritic cells. Eur J Immunol. 2013; 43:1862-72.
29. Wehner R, Wehrum D, Bornhauser M, Zhao S, Schakel K, Bachmann MP, et al. Mesenchymal stem cells efficiently inhibit the proinflammatory properties of 6-sulfo LacNAc dendritic cells. Haematologica. 2009; 94:1151-6.
30. Hancharou AY, Antonevich NH, DuBuske LM. Induction of Tolerogenic Dendritic Cells UsingCo-Culture with Human Olfactory Mucosa-Derived Mesenchymal Stem Cells. J ALLERGY CLIN IMMUNOL. 2016; 137:115.
31. Kasai S, Inui M, Nakamura K, Kakizaki Y, Endo S, Nakamura A, et al. A novel regulatory role of gp49B on dendritic cells in T-cell priming. Eur J Immunol. 2008; 38:2426-37.