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TNF-α modulates the immunosuppressive effects of MSCs on dendritic cells and T cells

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ABSTRACT

Mesenchymal stem cells are progenitor cells that have capabilities to differentiate different cell types. Also, MSCs possess immune suppressive effects on DC differentiation and T cell activation through a wide range of soluble factors and receptors. The properties of MSCs change through activation of cytokines particularly IFN-γ and TNF-α. The DC phenotypes and functions including the expression of co-stimulatory and co-inhibitory molecules and capabilities of DCs to induce allogeneic activation of CFSE-labeled splenocytes as well as cytokine production when they were differentiated in the presence of MSCs, TNF-α activated MSCs, IFN-γ activated MSCs and IFN-γ & TNF-α activated MSCs were analyzed. Treg population and T cell polarization were investigated using flowcytometry and real-time PCR respectively. Here, we showed that IFN-γ slightly enhances immunosuppressive effects of MSCs on immune system through induction tolerogenic DCs with elevated expression of IDO and increasing Treg population. Conversely, TNF-α decreases immunomodulation properties of MSCs on immune cells through the enhancement of co-stimulatory molecules such as ICOSL and HLA-DR, reduction of PDL-1 and PDL-2 expression and decrease of TGF-β and IL-10 in DCs as well as inhibition of T cell polarization into T_H2 and Treg. Taken together, these data showed crucial effects of microenvironments on MSC behaviors indicating that functions of MSCs differentially altered in the presence of different cytokines.

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1. Introduction

Dendritic cells are considered as main regulators of primary and secondary immune responses. DCs play central roles to orchestrate of adaptive immune responses and tolerance to self-antigens through co-stimulatory and inhibitory receptors as well as cytokine production [1,2]. The dual functions of DCs are related to their maturation state. Specific micro-environment in DC differentiation results to generate distinct DCs with different functional capabilities [3,4]. Conventional DCs shifted toward tolerogenic DCs when cultured in presence of MSCs [5,6].

Mesenchymal stem cells are multi-potential progenitor cells can differentiate into different lineage of cell types including adipocyte, chondrocyte and osteocyte. MSCs are isolated from diverse tissues such as bone marrow, placenta, amnion, cord blood and adipose tissues. Also, MSCs have characterized using adherence to plastic, expression of specific marker such as CD90, CD29, CD73, CD105, negatively expression of CD45 and CD34 markers. MSCs functions, cytokine production, receptors expression and proliferation differentially modulate by cytokines [7].

Several studies have shown that MSCs play important roles in differentiation, maturation and polarization of immune cells such as DCs and naive T cells through cytokine, growth factors and molecule production as well as expression of receptors on their surfaces [8,9]. MSCs suppress T cells functions and increase Treg populations. Furthermore, MSCs inhibit TH1 and TH17 cells generation [10]. Also, tolerogenic DCs are induced by MSCs [11]. Induction of immunosuppressive effects from MSCs need to prior activation of these cells by secreted cytokines such as IL-10 and IFN-γ from immune cells. After activation, MSCs can secrete battery of soluble factors such as IL-6, prostaglandin E2 and indoleamine 2, 3-deoxigenase (IDO). All of these released factors have immunomodulation effects on immune cells [12]. Expression of co-stimulatory molecules on DCs decreases by IL-6 and thereby inhibits T cells priming eventually [11]. Notably, some researches showed the anti-tumor effects of MSCs upon activation of MSCs by special cytokines such as TNF-α. Lee et al. indicated that pre-activation of MSCs with TNF-α lead to enhance anti-tumor properties of MSCs [13]. Also, TNF-α induces expression of ICAM-1 in MSCs with p53-dependent manner resulting to increase MSCs migration [14]. These data indicated that effects of MSCs are changed in presence of different cytokines.

With regard to diverse behaviors of MSCs in different microenvironment and little data on effects of TNF-α and TNF-α plus IFN-γ on immunosuppressive effects of MSCs, we analyzed the DC differentiation along with T cell functions and its polarization in presence of MSCs

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Table 1
Sequences of oligonucleotide primers used for amplification in real-time PCRs.

Gene	Sequence	
	Forward	Reverse
IFN-γ	ATT ACT GAG GGG TGT C	CGA ATC AGC AGC GAC TCC
IL-17	TGG GAT TAC AAC ATC ACT CG	AGG ATT TCT TGC TGA ATG G
T-bet	AGC AAG GAC GGC GAA TG	GGTGGA CAT ATA AGC GGT TC
GATA-3	TGA CGG AAG AAG TGG ACG	CTG GCT CCC GTG GTG G
ROR-γ	CAA ATA CGG TGG TGT GGA G	ACG GTT GGC ATT GAT GAG
Foxp3	GGT ATT GAG GGT GGG TGT C	AGG CAG GCT GGA TAA CG
IDO	GTACATCACCATGGCGTATG	CGAGGAAGAAGCCCTTGTC
GAPDH	GGT GAA GGT CGG TGT GAA CG	CTC GCT CCT GGA AGA TGG TG

and pre-activated MSCs with different cytokines including TNF-α (TNF-α–MSC), IFN-γ (IFN-γ–MSC) and TNF-α + IFN-γ (TNF-α & IFN-γ–MSCs).

2. Material and methods

2.1. Animals

Female Balb/c and DBA mice were purchased from Pasteur Institute of Iran (Tehran, Iran) and used at 6–8 weeks old.

2.2. MSC culture and characterization

Murine adipose-derived MSCs (AD-MSCs) were isolated from BALB/c mice according to previous studies [15]. Briefly, the extracted inguinal adipose tissues were digested using collagenase type I (1 μg/mL) (Gibco) following sacrifice of 6–8 old mice. Then, adipose tissue-derived cell suspension were cultured into T-75 plastic cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented by 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin (all from Gibco) at 37 °C and 5% CO_2. After 24 h, the non-adherent cells were removed. The adherent cells were expanded till passage 3 (P3). The expanded cells at passage 3 were assessed for expression a battery of markers including CD73, CD105, CD90 and CD29 expression and negative for CD11b and CD45 markers using flow cytometry (all antibodies from eBioscience, San Diego, CA, USA). The specific conditioned medium was used to evaluate the differentiation capacity of the expanded cells according to previous studies.

2.3. MSC treatment

Murine adipose derived MSCs (AT-MSCs) were treated by TNF-α (10 ng/mL), IFN-γ (10 ng/mL) and TNF-α (5 ng/mL) plus IFN-γ (5 ng/mL) (all from eBioscience) for 48 h. After incubation, the expression of specific surfaces markers and differentiation capacity were analyzed by flow cytometry and specific conditioned medium respectively.

2.4. Bone marrow cells Isolation

Bone marrow cells (BM) were isolated from 6 to 8 weeks Balb/c mice by flushing femurs using 2 mL phosphate-buffered saline (PBS) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Life Technologies). To lyse red blood cells, the BM cells were suspended in tris-ammonium chloride at 37 °C for 2 min. The remained cells were re-suspended in RPMI1640 Media to dendritic cells differentiation

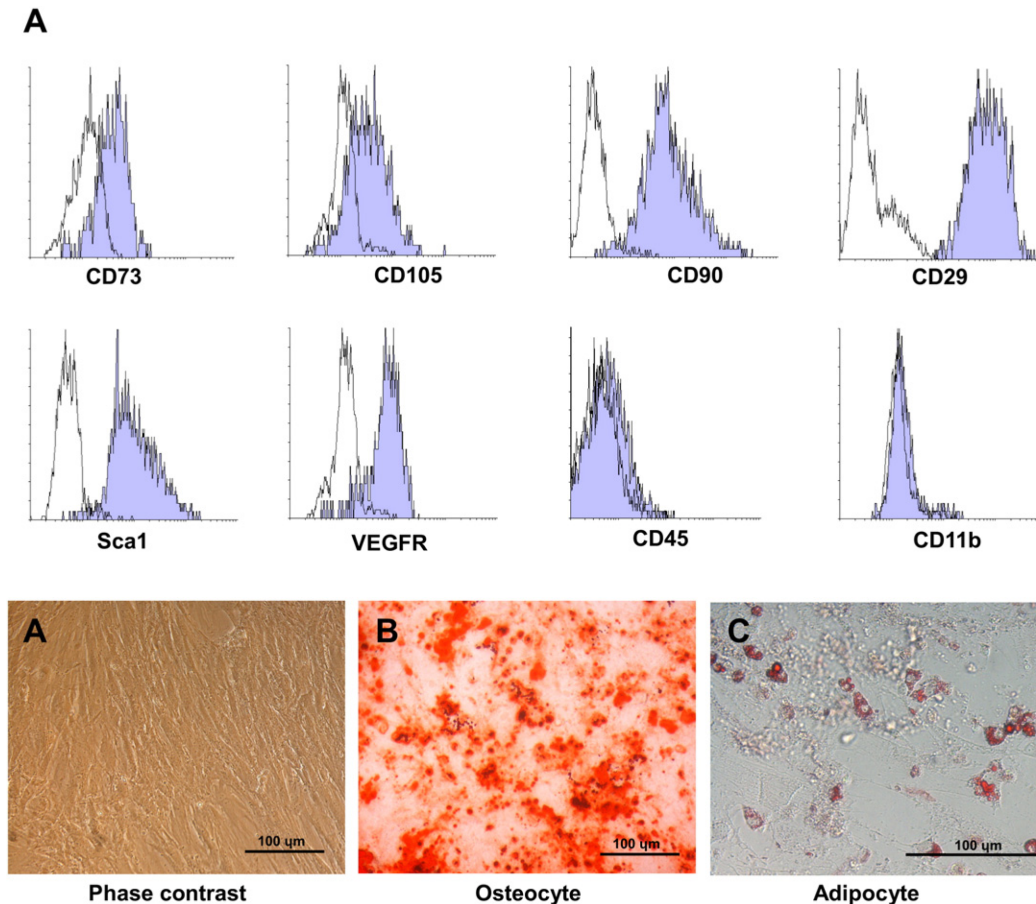

Fig. 1. Characteristics of adipose tissue derived mesenchymal stem cell. (A) Microscopic image of representative example of MSCs (left below). (A) Representative example of flow cytometric analysis of cell surface marker expression on freshly isolated MSC (above). (B) Differentiation of MSCs to osteocyte in Alizarin Red medium. (C) Differentiation of MSCs to adipocyte in Oil red medium.

supplemented with essential and nonessential amino acids, 1 mmol/L sodium pyruvate, 2.5 mmol/L Hepes buffer pH 7.4, 50 μmol/L 2-mercaptoethanol (2-ME), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.3 mg/mL L-glutamine, and 10% FBS (from Gibco).

2.5. Cell culture for DC differentiation

BM cells were cultured in Dendritic Culture Media containing 20 ng/mL mouse GM-CSF and 7 ng/mL mouse IL-4 (Peprotech) at concentration 1×10^6/mL in 6-well plates (SPL) for 6 days. Half of the medium was change every 3 days with fresh DC media. Cultured cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. DCs were harvested from the cultures by gently pipetting and removing non-adherent cells, then each wells was washed 2 times with PBS to remove suspend cells. At day 5, immature BM derived DCs were cultured on MSC groups including MSCs, MSCs pre-treated with TNF-α, MSCs pre-treated with IFN-γ and MSCs pre-treated with TNF-α plus IFN-γ at 10:1 ratio until day 7. Control group were cultured in absence of MSCs with same culturing condition of MSCs groups.

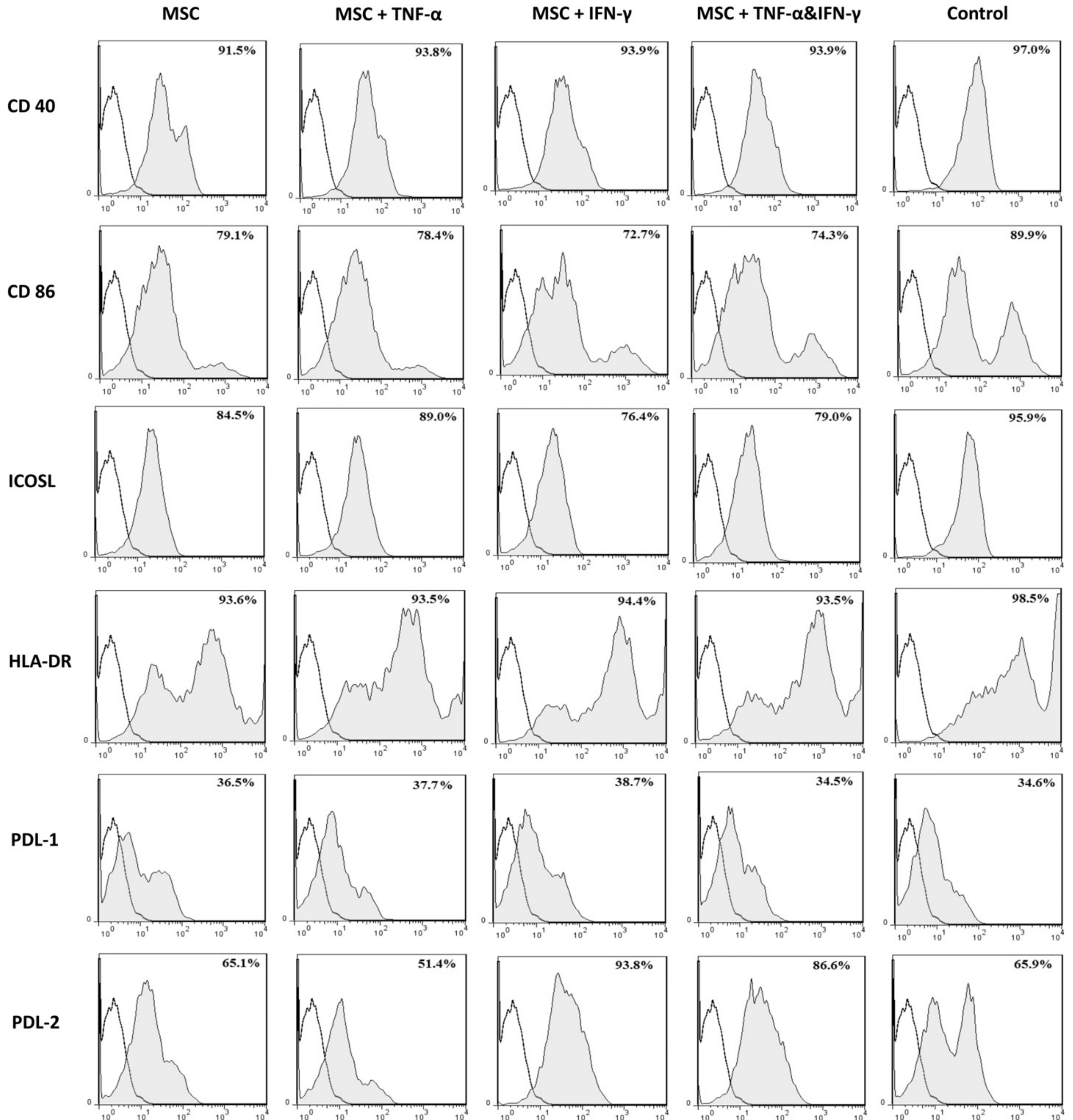

Fig. 2. The expression of co-stimulatory and co-inhibitory molecules on BM derived DCs in presence of cytokines pre-activated MSCs. Control represents data of conventional BM derived DCs.

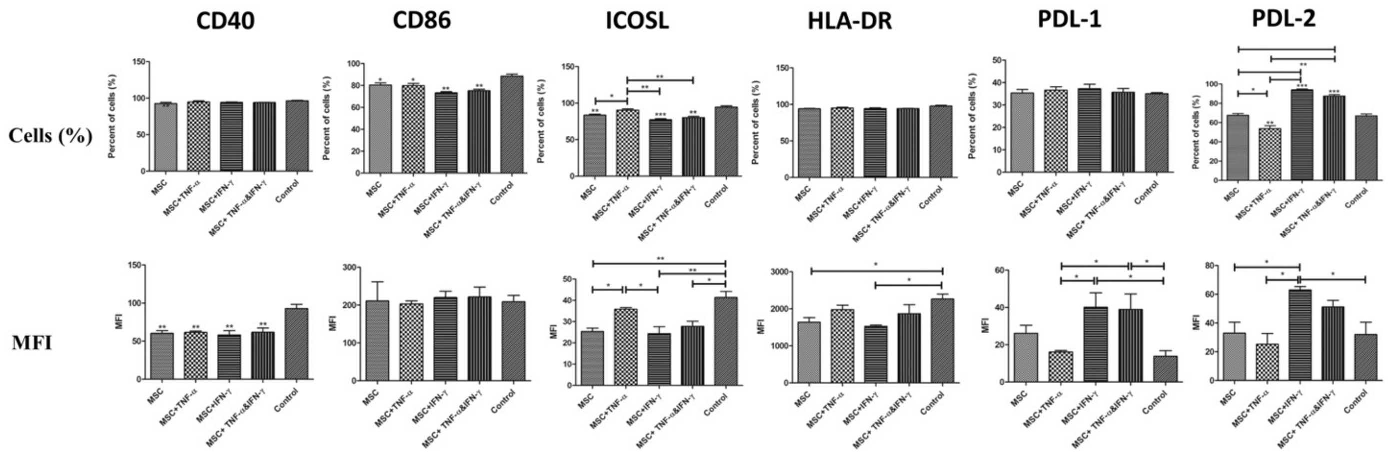

Fig. 3. Expression of co-stimulatory molecules including ICOSL and HLA-DR on DCs significantly decreased in presence of diverse cytokines treated MSCs excluding TNF-α activated MSCs. On other hands, pre-activated MSCs except TNF-α-MSCs remarkably increased the expression of co-inhibitory markers including PDL-1 and PDL-2 on BM derived DCs. Expression of CD40 meaningfully reduced on DCs in presence of both MSCs and cytokines activated MSCs. Control represents data of conventional BM derived DCs. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

2.6. Flowcytometric analysis

At day 7, DCs were harvested and centrifuged once and re-suspended in cell-staining medium (SM) consisting of PBS supplemented with 2% heat-inactivated calf serum (Gibco), 2% heat-inactivated mouse serum, 10 mg/mL 2.4G2 anti-Fc receptor mAb, and 0.02% sodium azide (Sigma) in all groups. Cells were blocked with SM at 4 °C for 20 min before incubation with mAbs. Cells were stained with conjugated mAbs for 35 min at 4 °C at 1×10^6 cells per sample in a 100-mL volume. The following mAbs were used: Pan DC marker (CD11c)-FITC, Rat IgG2a K Isotype Control-APC and PE conjugated CD40, CD86, ICOSL, HLA-DR, PDL-1 and PDL-2 markers. All Antibody provided from Ebioscience. Flow cytometry analysis was done using BD FACS CanII and Flowjo software.

2.7. Regulatory T cells analysis

The isolated splenocyte cells were isolated from Balb/c mice and co-culture with MSCs, MSCs pre-treated with TNF-α, MSCs pre-treated with IFN-γ and MSCs pre-treated with TNF-α plus IFN-γ in presence of PHA (2.5 μg/mL) for six days at 6-well plates. At day 7, the Treg population was analyzed using Treg Mouse Regulatory T Cell Staining Kit (Ebioscience, USA) containing FITC-CD4, PE-CD25 and APC-Foxp-3 antibodies. The untreated splenocytes group was considered as control group.

2.8. Mixed lymphocyte reaction

Mixed lymphocyte reaction were performed using CFSE labeled splenocytes isolating from DBA inbred mice which were cultured with MSCs treated DCs in different groups. MLR assay were evaluated by culturing of mentioned DCs in triplicate at concentration 10^4 cells/well in a 96-well round-bottom plates with 10^5 splenocytes/well for 48 h. The proliferation of splenocyte responder cells was analyzed by flow cytometry using BD FACS CanII and Flowjo software. Control group was the conventional DCs without any treatment.

2.9. ELISA

The supernatants of DCs co-cultured with MSCs in diverse groups as well as control groups were collected at day 7 and cytokine measurements were done for TGF-β, TNF-α, IL-10 and using an enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocol. Also, isolated splenocytes from Balb/c mice were co-cultured with MSCs, MSCs pre-treated with TNF-α, MSCs pre-treated with IFN-γ and MSCs pre-treated with TNF-α plus IFN-γ with 10:1 ratio in presence of PHA (2.5 μg/mL) for 72 h. After three days, the released cytokines

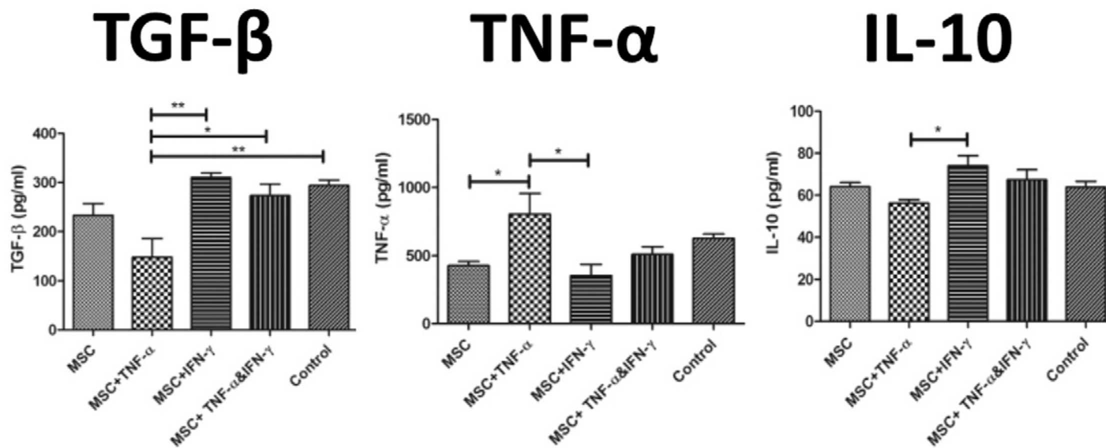

Fig. 4. The cytokine production of differentiated DCs in absence or presence of MSCs or cytokines activated MSCs. The releasing of TGF-β and IL-10 showed alleviations in BM derived DCs in presence of MSCs and cytokines activated MSCs excepting TNF-α treated MSCs. Otherwise, TNF-α treated MSCs significantly increased TNF-α secretion by DCs compared to MSCs and IFN-γ-MSC groups. Control represents data of conventional BM derived DCs. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

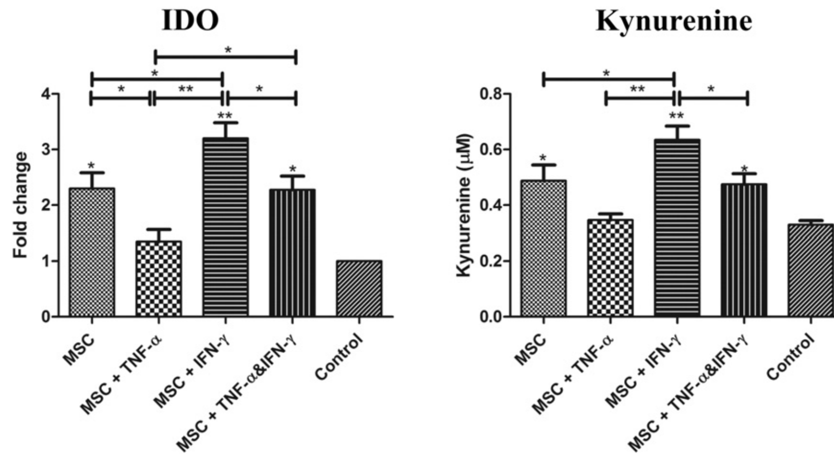

Fig. 5. The capabilities of BM-DCs in absence or presence of MSCs and cytokines activated MSCs to induce the allogeneic activation of T cells using CFSE labeled splenocytes. Control represents data of conventional BM derived DCs.

including TGF-β, TNF-α, IL-10 by splenocytes were measured using ELISA.

2.10. Relative quantitative real-time RT-PCR

Total RNA from the MSCs treated splenocytes and untreated splenocytes were prepared using an RNA extraction kit (TRIzol, Invitrogen) according to manufacturer's protocol. cDNA was synthesized by Sensiscript® Reverse Transcription Kit (QIAGEN, Hilden, Germany) from total RNA and stored at −80 °C until use. Relative quantitative reverse transcriptase PCR was performed to quantify the expression of IFN-y, IL-17, IDO, T-bet, ROR-γ, GATA-3 and Foxp-3 mRNA using Real qPCR 2× Master Mix SYBR Green (Ampliqon) according to the manufacturer's protocol on Qiagen/Corbett Rotor-Gene® Q light cycler. The primer sequences are summarized in Table 1.

2.11. Enzyme assay of IDO activity

IDO activity was evaluated according to previous study [16]. Briefly, the proteins of culture supernatant of DCs and MSCs conditioned DCs were precipitated by 15% perchloric acid. About 25 μL of clear supernatant was injected onto a high-pressure chromatography (ACTA Purifier, Pharmacia, Sweden) with C18 ion exchanger column. Kynurenine was detected by a UV-detector at a wavelength of 360 nm.

2.12. Statistical analysis

Statistical analysis was carried out with GraphPad Prism version 4.0 and SPSS 16.0. Two-tailed Student's *t*-test and ANOVA with post-hoc Tukey's Multiple Comparison tests were used for differences between two groups and more than two groups respectively. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytokines treated MSCs differentially changed the expression of co-stimulatory and inhibitory molecules on DCs

We first documented and characterized the murine AT-MSCs before the evaluation effects of TNF-α, IFN-γ and TNF-α plus IFN-γ on proliferation capacity, pluripotency genes and immunomodulary factors. All mouse MSCs derived from healthy adipose exhibited typical spindle-shaped morphology and were positive for MSC markers including CD73, CD105, CD90 and CD29; and negative for CD11b, Sca-1, CD45 and VEGFR markers (Fig. 1).

DCs were differentiated from bone marrow of mice according to standard operating procedure until day 5. At this time, the normal culture medium was replaced by MSC cells in diverse groups including MSCs, MSCs pre-treated with TNF-α, MSCs pre-treated with IFN-γ and MSCs pre-treated with TNF-α plus IFN-γ during the differentiation and maturation of DCs. The conventional assay to characterize the phenotype of derived DCs in presence of MSCs and pre-treated MSCs were performed as shown in Fig. 2. The expression of co-stimulatory and inhibitory molecules on differentiated DCs differentially changed in both percent of positive cells and mean intensity fluorescence (MFI). We observed significant reduction of ICOSL positive cells on DCs which differentiated in presence of MSCs, MSCs pre-treated with IFN-γ and MSCs pre-treated with TNF-α plus IFN-γ. Also, PDL-2 positive derived DCs increased when co-cultured with MSCs, MSCs-IFN-γ and MSCs-TNF-α& IFN-γ (Fig. 3 *upper panel*). Interestingly, treated MSCs with TNF-α (MSCs-TNF-α) remarkably decreased the PDL-2 positive cells, but had no effect on ICOSL positive derived DCs (Fig. 3 *upper panel*). Furthermore, the expression (MFI) of co-stimulatory and inhibitory molecules on derived DCs changed. The expression of CD40, CD86, ICOSL and HLA-DR significantly diminished when DCs differentiated in

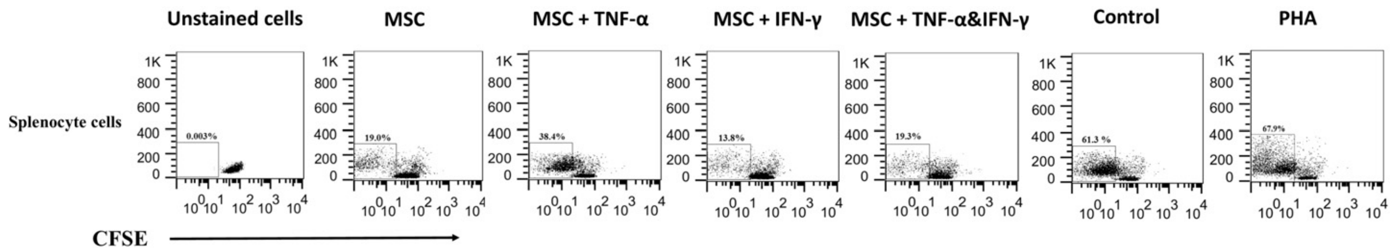

Fig. 6. (A) statistical analysis represented a significant reduction in abilities of DCs when they differentiated in presence of MSCs and cytokines activated MSCs. But, reduced capabilities of derived DCs in presence of TNF-α-MSCs were significantly less than DCs when they differentiated in presence of IFN-γ-MSCs. (B) Treg frequency was analyzed in PHA-activated MSCs upon co-cultured with MSCs and cytokine activated MSCs. Results showed a significant increase the Treg frequency in presence of MSCs and cytokines treated MSCs excepting TNF-α treated MSCs. Control represents data of conventional splenocytes culturing. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

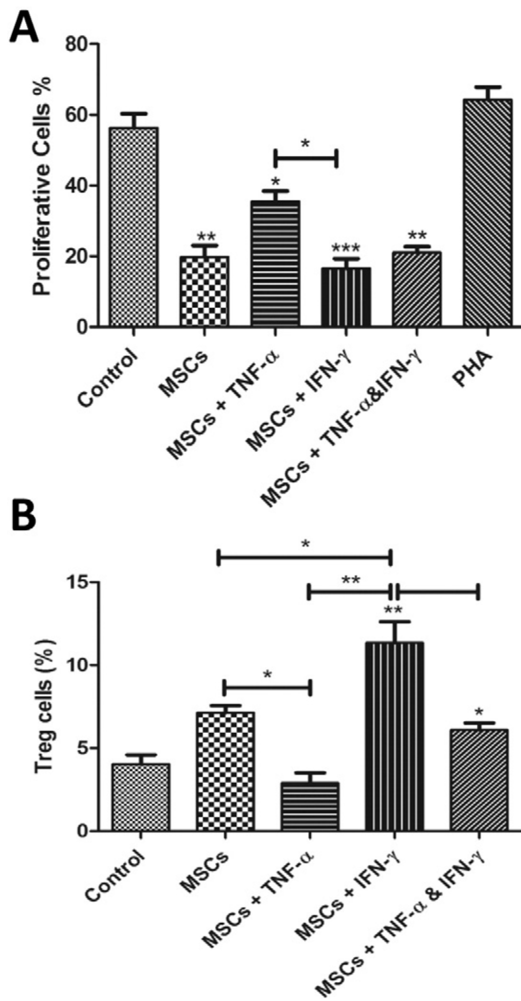

Fig. 7. IDO expression in DCs following co-culture with MSCs and activated MSCs. (A) IDO expression significantly increased in MSCs conditioned DCs except TNF-α treated MSCs group when compared to control group (Conventional DCs). IFN-γ treated MSCs remarkably induced IDO expression in DCs in comparison with MSCs and TNF-α&IFN-γ treated MSCs. (B) Kynurenine production in DCs as product of IDO enzyme activity. Kynurenine production noticeably increased in MSCs conditioned DCs except TNF-α treated MSC group when compared to control group. Kynurenine production remarkably increased in IFN-γ + MSCs conditioned DCs when compared with MSCs and TNF-α&IFN-γ treated MSCs groups. Control represents data of conventional BM derived DCs. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

presence of MSCs, MSCs-IFN-γ and MSCs-TNF-α& IFN-γ (Fig. 3 *lower panel*). Also, we observed that the expression of inhibitory molecules such as PDL-1 and PDL-2 noticeably increased in MSCs, MSCs-IFN-γ and MSCs-TNF-α& IFN-γ groups (Fig. 3 *lower panel*). Notably, the expression of co-stimulatory and inhibitory molecules did not change when DCs differentiated in the presence of MSCs-TNF-α except CD40 ligand (Fig. 3 lower panel). We detected a significant decrease of the expression of CD40 on DCs in MSCs-TNF-α group.

3.2. Cytokine production altered in MSCs treated DCs

The cytokine production by DCs in different groups was analyzed using ELISA. Obtained data showed a significant increase of TGF-β and IL-10 production in DCs that treated with MSCs-IFN-γ compared to MSCs-TNF-α group (Fig. 4). Also, we observed that presence of TNFα treated MSCs leads to meaningfully reduce TGF-β secretion in derived DCs compared to untreated DCs (Fig. 4). Conversely, the secretion of TNF-α in remarkably increased when DCs differentiated in presence of TNF-α treated MSCs in comparison to MSCs and IFN-γ treated MSCs groups (Fig. 4).

3.3. IDO expression and function increased in MSC conditioned DCs except in TNF-α treated MSCs

IDO expression in MSCs conditioned DCs in different groups was evaluated using real-time PCR (Fig. 5). The obtained data showed that expression of IDO significantly increased in MSC conditioned DCs except in TNF-α treated MSCs in comparison with untreated DCs (control) and TNF-α-MSCs conditioned DCs (TNF-α + MSCs) ($P < 0.05$). Also, the expression of IDO in IFN-γ-MSCs conditioned DCs significantly increased in comparison with MSCs and TNF-α + IFN-γ treated MSCs groups ($P < 0.05$). We also analyzed the production of kynurenine as product of IDO enzyme by degradation of tryptophan (Fig. 5). Our results showed that the production of kynurenine significantly increased in DCs co-cultured with cytokines treated MSCs except TNF-α + MSCs group in comparison with conventional DCs (control) ($P < 0.01$). The production of kynurenine significantly enhanced in IFN-γ-MSC conditioned DCs in comparison with DCs which were co-cultured with MSCs and TNF-α + IFN-γ treated MSCs ($P < 0.05$).

3.4. Capabilities of MSCs treated DCs changed to induce T cells proliferation regarding to pre-activation of MSCs with cytokines

The capacity of derived DCs to stimulate allogeneic T cells was assessed by co-culturing of CFSE labeled splenocytes (Fig. 6). The results showed a significant reduction in splenocytes proliferation in MLR on MSCs derived DCs in all groups. (Fig. 7 A). But, capabilities of derived DCs in presence of TNF-α treated MSCs to stimulate splenocytes significantly increased compared to MSCs-IFN-γ group (Fig. 7 A).

3.5. Treg population not changed in presence of TNF-α treated MSCs

To analysis Treg population, splenocytes were cultured in presence of MSCs, MSCs pre-treated with TNF-α, MSCs pre-treated with IFN-γ and MSCs pre-treated with TNF-α plus IFN-γ for six days. The splenocytes

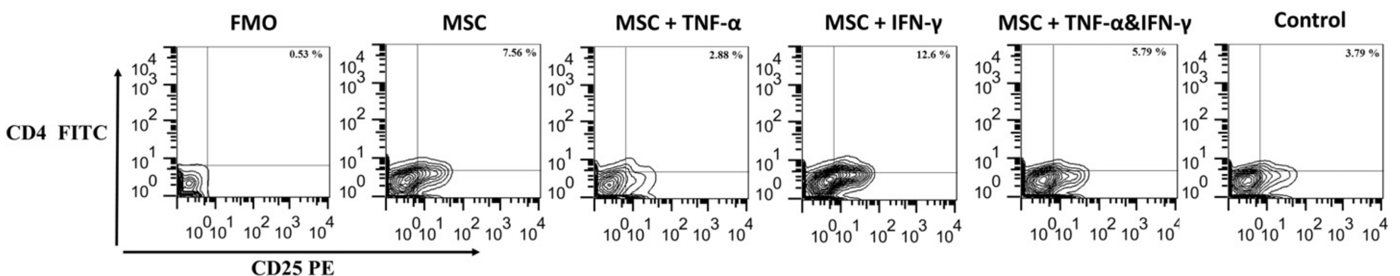

Fig. 8. Regulatory T cell frequency analysis in PHA-activated splenocytes upon culturing with MSCs and activated MSCs using flowcytometry after six days. Notably, at day 7, we did not detect the Foxp3 protein by APC-Foxp3 antibody and so, we analyzed Treg population with FITC-CD4 and PE-CD25 antibodies. Control represents data of conventional splenocyte culturing.

Fig. 9. The production of cytokines and expression of T cell subtypes transcription factors in PHA-activated splenocytes in absence or presence of MSCs and activated MSCs. The secretion of TGF-β and IL-10 showed remarkable alleviations in PHA-activated splenocytes in presence of MSCs and cytokines activated MSCs excepting TNF-α treated MSCs compared to control and TNF-α-MSCs groups. Otherwise, TNF-α treated MSCs significantly increased the secretion of TNF-α activated splenocytes compared to IFN-γ-MSCs group. Also, presence of MSCs and activated MSCs excluding TNF-α treated MSCs leads to polarize of naïve T cells toward T_H2 and Treg subtypes through a significant enhancement of GATA-3 and Foxp3 expression as well as reduction of T-bet and ROR-γ as main transcription factors in T_H1 and T_H17 polarization. Control represents data of conventional splenocytes culturing. (*: P < 0.05, **: P < 0.01, ***: P < 0.001).

were activated by PHA. Interestingly, Foxp-3 molecules were not detected after six days and then we analyzed the Treg population based on CD4 and CD25 according to previous studies (Fig. 8). Flowcytometric analysis showed that Treg frequency significantly increased when splenocytes cultured in presence of MSCs, MSCs-IFN-γ and MSCs-TNF-α&IFN-γ (Fig. 7 B). Notably, the frequency of Treg showed no changes in MSCs-TNF-α group (Fig. 7 B).

3.6. Pre-activation of MSCs with different cytokines results to change cytokine production and T cell polarization in splenocytes

The splenocytes were co-cultured with MSCs and different treated MSCs for three days and then the production of TGF-β, TNF-α and IL-10 as well as relative expression of transcription factors and cytokines including T-bet, ROR-γ, GATA-3, Foxp-3, IFN-γ and IL-17 were analyzed.

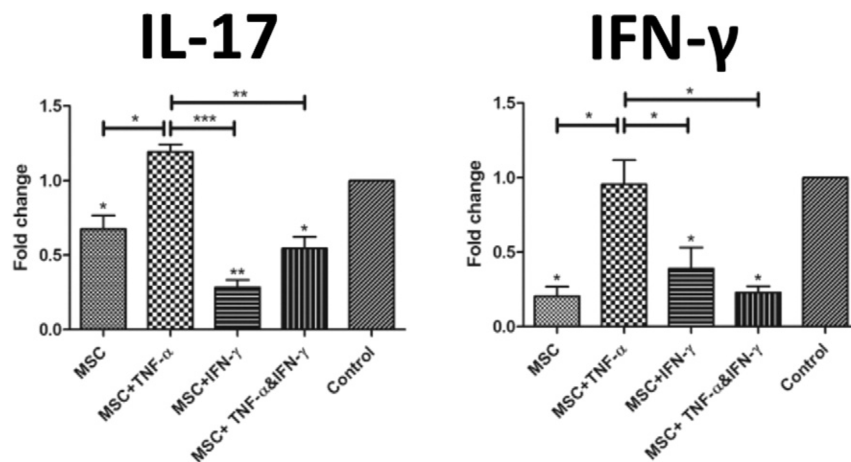

Fig. 10. Expression of IFN-γ and IL-17 mRNA in PHA-activated splenocytes in presence of MSCs and activated MSCs. The expression of both IFN-γ and IL-17 decreased in activated splenocytes in presence of MSCs or activated MSCs excepting TNF-α activated MSCs. Control represents data of conventional splenocytes culturing. (*: P < 0.05, **: P < 0.01, ***: P < 0.001).

Results showed that MSCs, MSCs-IFN-γ and MSCs-TNF-α&IFN-γ cause to increase the production of TGF-β and IL-10 in splenocytes (Fig. 9 A). On other hands, TNF-α secretion remarkably diminished in splenocytes after co-culture with IFN-γ treated MSCs (Fig. 9 A). Conversely, we observed no changes in cytokine production of splenocytes in presence of MSCs-TNF-α. Furthermore, relative expression of IFN-γ and IL-17 noticeably down-regulated in splenocytes cultured with MSCs, MSCs-IFN-γ and MSCs-TNF-α&IFN-γ without no significant changes in TNF-α-MSCs treated splenocytes (Fig. 10). Also, relative expression analysis of transcription factor revealed that T-bet and ROR-γ significantly decreased in splenocytes cultured in presence of MSCs in all groups except TNF-α treated MSCs. In contrast, the expression of Foxp-3 and GATA-3 mRNA in splenocytes when these co-cultured with MSCs and IFN-γ treated MSCs (Fig. 9 B).

4. Discussion

MSCs are present in many tissues especially in the bone marrow. During inflammation, MSCs circulate in bloodstream and migrate to inflamed and malignant tissues. Furthermore, the cellular development and biological function of different types of immune cells in innate and adaptive immune system are regulated by MSCs [17,18]. MSCs exert their immunomodulation properties through secretion of various cytokines as well as expression of different receptors and molecules. Previous studies indicated that local microenvironment determine the fate both MSCs and MSCs interacted cells [19]. Immunosuppressive effects of MSCs have shown in several investigations. MSCs inhibit DCs differentiation, migration and capability to prime T cells partially mediated by releasing of IL-6, IL-10 and PGE2 [20]. PGE2 induced IDO expression in DCs and subsequently inhibits the capacity of DCs to stimulate anticancer immune response [21]. Moreover, TGF-β increased IDO expression in DCs [22]. So, increased levels of IDO in MSCs conditioned DCs are possibly related to secretion of PGE2 and TGF-β. More recently, it has shown that MSCs need to prime with IFN-γ for applying their immunosuppressive effects. Also, the MSC functions revealed to be mediated by soluble factors and expressed molecules such as IDO and PDL-1 induced by IFN-γ [12,19]. MSCs or activated MSCs excluding TNF-α-MSCs exhibit a tolerogenic phenotype. On the other hand, PDL-1 and PDL-2 expressions were enhanced. In agreement with these co-stimulatory and inhibitory molecule changes, the capacity of DCs which generated in presence of MSCs induces activation of splenocytes decreased. These immunosuppressive effects of MSCs on DC phenotype and functions are likely associated to secretion of soluble factors from MSCs such as GRO-γ, IDO and IL-10 [5,6,23]. Other investigations reported that MSCs induce tolerogenic DCs through IL-6 and GM-GSF production [6]. But, we did not observe inhibitory effects of MSCs on differentiation of DCs from monocyte that was previously reported.

Both IFN-γ and TNF-α cytokines are crucial pro-inflammatory cytokines that differentially regulated MSC functions. Previous studies revealed that TNF-α increases anti-tumor effects of BM-MSCs through over expression TRAIL and DKK-3 [13]. Also, TNF-α not induced IDO production and PDL-1 expression by MSCs. Furthermore, TNF-α enhances the migratory properties of MSCs through the alleviation of ICAM-1 expression. In contrast, IFN-γ induced IDO production by MSCs. IDO secreted from MSCs plays critical role to inhibit MLR. Notably, IFN-γ induced the expression of PDL-1 on MSCs. PDL-1 was initially reported as inhibitory molecules restricting to lymphoid cells including T cells, B cells, macrophages and DCs. But, some reports indicated that PDL-1 also express by endothelial cells. Several investigations revealed that inhibition of lymphocyte proliferation by MSCs mediated by PDL-1 molecule [12,14,19,24].

Here, we observed that the enhancement of TGF-β, IL-10 and decreasing of TNF-α in differentiated DCs in presence MSCs, MSCs-IFN-γ and MSCs-IFN-γ-TNF-α. These data are similar to previous study reported by Chen et al. that revealed that MSCs switch the development of monocyte derived DCs toward DCs with immunosuppressive phenotype through secretion of growth regulated oncogene-γ (GRO-γ) [23]. So, lower level of TGF-γ and IL-10 in derived DCs in presence of TNF-α treated MSCs may related to decrease of GRO-γ production by TNF-α in MSCs.

We also analyzed the effects of MSCs or cytokine pre-activated MSCs on Treg frequency and T cell polarization. Obtained data showed that MSCs or pre-activated MSCs except TNF-α treated MSCs polarize the splenocytes toward Treg and T_H2 phenotypes and decreases T_H1 and T_H17 T cells. Based on previous reports, MSCs inhibit the differentiation of naïve T cells to T_H17 cells through IDO, TGF-β and PGE2 [25]. Also, MSCs induce trimethylation at promoter of Foxp3 resulting to suppress of ROR-C trimethylation through IL-10 production. These evidence lead to decrease T_H17 as well as increase of Treg frequencies [26]. Enhanced immune suppressive effects of IFN-γ treated MSCs are partially associated to induce of IDO and PGE2 production in MSCs by IFN-γ. TNF-α induces no change on IDO expression and low levels of TGF-β compared to IFN-γ.

In summary, present results showed a significant changes in MSCs immunosuppressive effects in presence of cytokine treated MSCs. As shown, TNF-α decreases or somewhere blocks the immunomodulation properties of MSCs. Conversely, suppression of immune cells has induced by IFN-γ. MSCs properties almost remain untouched in presence of both of IFN-γ and TNF-α together showing the contradiction effects of TNF-α and IFN-γ on MSCs. Notably, in presence of both cytokines, effects of IFN-γ were dominant compared to TNF-α.

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