

# The Role of TNF- $\alpha$ induced MSCs on Suppressive Inflammation by Increasing TGF- $\beta$ and IL-10

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## Abstract

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**BACKGROUND:** Mesenchymal stem cells (MSCs) may serve as immunoregulators by producing various anti-inflammatory molecules. Under sufficient level of TNF- $\alpha$ , MSCs become activated and adopt immune-suppressive phenotype (MSCs type-2) by releasing various anti-inflammatory molecule including TGF- $\beta$  and IL-10. However, the ability of MSC itself to produce IL-10 under TNF- $\alpha$  stimulation and the correlation of TGF- $\beta$  production of MSCs to IL-10 level remains to be elucidated.

**AIM:** In this study, MSCs were activated with various TNF- $\alpha$  doses to determine the increase of IL-10 and TGF- $\beta$  level as well as its correlation.

**MATERIAL AND METHODS:** This study used post-test only control group design, by using 3 study groups, consist of 1 control (C) and 2 treatments (T) (TNF- $\alpha$  = 5 and 10 ng/mL) with triplicate induced in MSC for 24 hours, then the levels of IL-10 and TGF- $\beta$  were measured by using ELISA assay.

**RESULTS:** The results of this study showed a significant increase of TGF- $\beta$  and IL-10 levels ( $p < 0.05$ ) at TNF- $\alpha$  5 and 10 ng/mL dose of TNF- $\alpha$ . Moreover, there was a significant negative correlation between TGF- $\beta$  and IL-10 level on 5 and 10 ng/mL dose TNF- $\alpha$  treatment.

**CONCLUSION:** Based on our study, we conclude that the 5 ng/mL dose of TNF- $\alpha$  is a sufficient dose for MSCs to suppress the inflammatory milieu. The higher increase of TGF beta is due to the controlled inflammation by IL-10.

## Introduction

Mesenchymal stem cells (MSCs) are classically defined as multipotent cells expressing the surface markers of CD73, CD90, CD105, and lacking the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and Human Leucocyte Antigen (HLA) class II. MSCs also can differentiate into osteocytes, chondrocytes and adipocytes under standard *in-vitro* differentiating conditions [1]. They can be isolated from the bone marrow, mobilised peripheral blood,

cord blood, umbilical cord (UC), placenta, adipose tissue, dental pulp, and even the fetal liver and lungs. On the other side, the autologous use has several limitations in decreased growth and [2] differentiation capacity [3] [4] of cell numbers and age-related changes. UC-MSCs show a gene expression profile more pluripotent and stemness than BM-MSCs [5] [6]. MSCs display profound immunomodulatory properties by suppressing excessive inflammatory responses of a variety of immune disorders.

Several studies have reported that MSCs actively interact and communicate with innate and

adaptive immune cells to ameliorate immune disorders [7] [8] [9]. Several clinical studies have shown that MSCs-based therapy effectively controls in various autoimmune disease, including Systemic Lupus Erythematosus (SLE), Graft versus Host Disease (GvHD), Rheumatoid Arthritis (RA), inflammatory bowel disease and multiple sclerosis.

Furthermore, the ability of immunosuppressive of MSCs is regarding the production of cytokines such as TGF- $\beta$ , IDO, NO, PGE<sub>2</sub>, IL-10 and TSG-6 [10] [11] [12]. IL-10 and TGF- $\beta$ 1 serve as potent anti-inflammatory cytokines in controlling excessive inflammatory responses. Specifically IL-10 attenuates pro-inflammatory signals by inhibiting pro-inflammatory cytokines release particularly IFN- $\gamma$ , IL-2, and TNF- $\alpha$  [13], while TGF- $\beta$ 1 ameliorates immune disorder by generating of CD4 + CD25 + FoxP3 + T<sub>reg</sub> [14].

TNF- $\alpha$  is an active stimulator molecule in enhancing the secretion of various inflammatory cytokines. The previous study reported that TNF- $\alpha$ -activated MSCs suppress inflammation by inducing IL-10 production in macrophage cells but MSCs its self were not the source of IL-10 due to MSCs from IL-10<sup>-/-</sup> mice were still effective in improving the survival of mice with sepsis [15]. A similar study has also described that IL-10 level decrease after 24 hours of intravenous infusion of mouse MSCs [16]. On the other side, TGF- $\beta$  as immunosuppressive molecules constitutively produced by MSCs [17] also involved in promoting the T-cell production of IL-10 through direct activation of IL-10 promoter via Co-Smad4 [18]. Under sufficient level of TNF- $\alpha$  MSCs become activated and adopt immune-suppressive phenotype (MSCs type-2) by releasing various anti-inflammatory molecule including TGF- $\beta$  and IL-10 [19]. However, the ability of MSC itself to produce IL-10 under TNF- $\alpha$  stimulation is yet unclear. Moreover, the correlation of TGF- $\beta$  production of MSCs to IL-10 level remains to be elucidated.

Therefore, in the present study, we explored the ability of MSCs in vitro in producing IL-10 and TGF- $\beta$  at 5 and 10 ng/mL TNF- $\alpha$  for 24-hour incubation.

## Material and Methods

Adult 19-day pregnant Wistar rats, weighing 350-450 g, were provided by the animal husbandry department. The animal was used according to good animal practices, and animal experiments were approved by our local animal care.

The umbilical cords were collected from the fetuses 19-day pregnant Wistar rats under general anaesthesia. The blood vessels were removed from

umbilical cord, then the tissue parts under aseptic conditions were cut into smaller pieces and transferred to a T25 culture flask containing DMEM (Sigma-Aldrich, Louis St, MO) supporting with 10% Fetal Bovine Serum (FBS) (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL)/streptomycin (100  $\mu$ g/mL) (Gibco™ Invitrogen, NY, USA). The UC tissues were incubated at 37°C in a humid atmosphere consisting of 5% CO<sub>2</sub>. The medium was renewed every 3 days, and after reaching 80% confluency (14 days), the cells were passaged. MSCs-like at passages 4–5 were used for the following experiments.

MSCs-like surface antigens were analysed by flow cytometric analysis at the fourth passage. The cells were subsequently incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated, Allophycocyanin (APC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies, including CD105, CD90 and CD73. FITC- APC- and PE-conjugated isotypes were used as negative controls. The analysis was performed using BD Pharmingen™ (BD Bioscience, Franklin Lakes, NJ, USA) at 4°C for 30 min. The cells were washed twice with 1% BSA/PBS, resuspended in 200  $\mu$ L 1% BSA/ PBS and analysed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

To characterise the isolated cells, we further performed the osteogenic differentiation assay at the fourth passage. Osteogenesis was induced by osteogenic induction medium containing 10 mmol/L  $\beta$  glycerophosphate, 10<sup>-7</sup> mol/L/ 0.1  $\mu$ M dexamethasone, 50  $\mu$ mol/L ascorbate-2-phosphate (Sigma-Aldrich, Louis St, MO) and supporting with 10% FBS (Gibco™ Invitrogen, NY, USA) in DMEM (Sigma-Aldrich, Louis St, MO) at 37°C and 5% CO<sub>2</sub>. Calcium deposition was shown by Alizarin Red staining (Sigma-Aldrich, Louis St, MO) after 21 days incubation.

MSCs (5 x 10<sup>4</sup> cells/well) was supplemented by TNF $\alpha$  recombinant (5 and 10 ng/mL (BioLegend, San Diego, CA)) in 24-well plate using DMEM (Sigma-Aldrich, Louis St, MO) then incubated for 24 hours at 37°C with 5% CO<sub>2</sub>. Each experiment was performed in triplicate. The TNF- $\alpha$  recombinant medium was collected and analysed for TGF $\beta$  and IL-10 levels using ELISA assay.

The levels of both TGF- $\beta$  and IL-10 released in the culture supernatants from the various treatment groups were measured by specific ELISA. Briefly, according to the manufacturer's instructions (Fine Test, Wuhan, China), TGF- $\beta$  and IL-10 were calculated according to a standard curve constructed for each assay, and each assay was performed in triplicate. The colourimetric absorbance was recorded at a wavelength of 450 nm.

Data are presented as the means  $\pm$  standard deviation. All calculations were carried out using IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used

for statistical analysis. The statistical significance of the differences between the groups was assessed using one way-ANOVA and continued with Duncan post-hoc analysis. Correlation between IL-10 and the TGF- $\beta$  level was done using one-tailed Pearson's test. *P* values: \*\*, *P* < 0.001.

## Results

Isolation of UC-MSCs was performed based on the capacity to plastic attachment under standard culture condition. Isolated cells were cultured for 2-3 weeks in monolayer and used for differentiation analysis after 4 to 5 passages. The UC-MSCs were initially characterized by their elongated fibroblastic cellular phenotype (Figure 1a). Moreover, Osteogenesis was confirmed at day 21 of culture by immunodetection with Alizarin Red staining (Figure 1b).

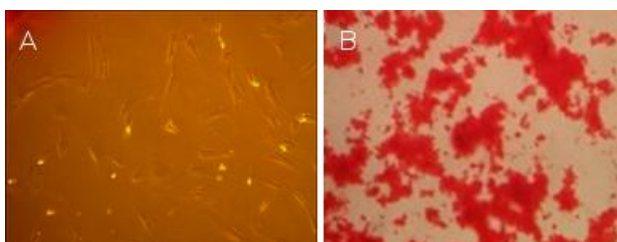


Figure 1: a) UC-MSCs characterisation was based on their peculiar fibroblast-like (spindle shape) morphology; b) and osteogenic differentiation with Alizarin Red staining appears red colour

The specific marker of UC-MSCs expression cultured in the media was evaluated as presented in Figure 2. We have characterised the expression pattern of UC-MSCs by flow cytometric analysis with the positive MSCs markers CD73, CD90 and CD105.

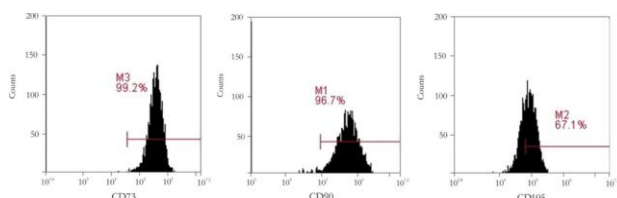


Figure 2: Detection by flow cytometric demonstrates positive expression of three UC-MSCs markers. Populations are 99.2% positive for CD73, 96.7% positive for CD90 and 67.1% positive for CD105

We subsequently quantify levels of IL-10 and TGF- $\beta$  (Figure 3) by ELISA in triplicate. The analysis of TGF- $\beta$  concentration showed that there was significantly increase in all treatments (*p* < 0.001) at 5 ng/mL ( $64.09 \pm 2.25$  ng/mL) and 10 ng/mL ( $92.78 \pm 1.28$  ng/mL) TNF- $\alpha$  dose. Furthermore, the IL-10 concentration on all treatments showed the significantly increased (*p* < 0.001) at 5 ng/mL ( $533.12$

$\pm 3.92$  ng/mL) and 10 ng/mL ( $513.42 \pm 4.31$  ng/mL) TNF- $\alpha$  dose. In other side, we found the negative correlation between TGF- $\beta$  and IL-10 level on 5 and 10 ng/mL dose TNF- $\alpha$  treatment (*p* = 0.007, *r* = -0.933).

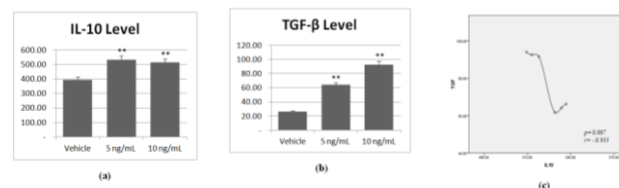


Figure 3: a) ELISA assays for two treatment groups with doses 5 and 10 ng/mL showed the highest concentration of TGF- $\beta$  level at 10 ng/mL TNF- $\alpha$  dose ( $92.78 \pm 1.28$  ng/mL), \*\*, *P* < 0.001; b) Furthermore, the optimum concentration of IL-10 level in MSCs medium was 5 ng/mL TNF- $\alpha$  dose ( $533.12 \pm 3.92$  ng/mL), \*\*, *P* < 0.001 vs vehicle control; c) Moreover, the data demonstrated that there was significant negative correlation between TGF- $\beta$  and IL-10 level on 5 and 10 ng/mL dose TNF- $\alpha$  treatment

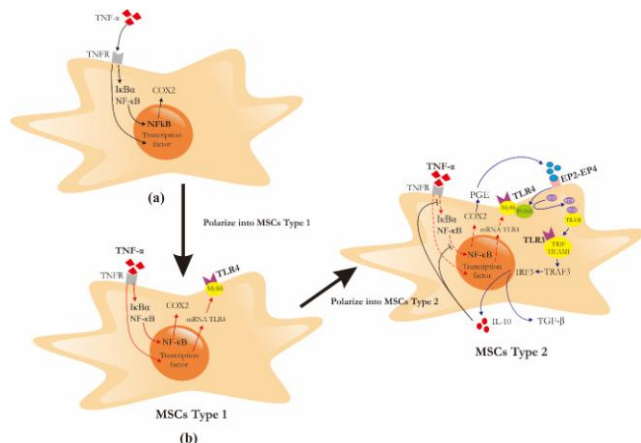
## Discussion

Inflammation is a cell protective response to eliminate various pathogens and preserve host integrity. MSCs as sensors of inflammatory may create both anti-inflammatory and proinflammatory effects when interacting to cell innate of the immune system or exposed by the various cytokine. Previous studies have shown that MSCs respond to inflammatory milieu by polarising either into MSCs type-2 with an immune-suppressive phenotype or MSCs type-1 with proinflammatory profile depend on Toll-Like Receptors (TLRs) type activation [7] [9] [21] [22]. Several studies have reported that immunosuppressive of MSCs occur through releasing various anti-inflammatory molecule including IL-10, IL1ra-1, PGE<sub>2</sub>, IDO, NO, TGF- $\beta$  and TSG-6. The release of its anti-inflammatory molecules may occur through co-cultures of MSCs along with immune cells or under TNF- $\alpha$ , IL-1 and IFN- $\gamma$  stimulation [15] [20]. The capacity of MSCs type-2 in suppressing the excessive inflammation and ameliorating immune disorders has opened new perspectives in clinical research, particularly in autoimmune disease.

Our studies demonstrated that MSCs released IL-10 level was significantly increase at 5 ng/mL and 10 ng/mL TNF- $\alpha$  (Figure 3a, *P* < 0.001). These data suggest that stimulation of low-dose TNF- $\alpha$  (5 ng/mL) can promote the polarisation of MSCs into MSCs type-2. Under sufficient dose of TNF- $\alpha$ , MSCs upregulate the expression of TLR-3 leading to release of the various anti-inflammatory molecule including IL-10 [8] [9]. The binding of TNF- $\alpha$  to TNFR-1 of MSCs resulted in the activation of NF- $\kappa$ B and ERK signalling which produces cyclooxygenase-2 (COX2) and upregulates TLR-4 expression. TLR4-primed MSCs population which are known as MSCs type-1 exhibit a proinflammatory profile, including upregulation of

COX2 which increase PGE<sub>2</sub> secretion. Specifically, PGE<sub>2</sub> bound to EP<sub>2</sub> and EP<sub>4</sub> receptors of MSCs leading to the shift from MyD88-dependent proinflammatory (MyD88-independent pathway) to TRIF-TRAM mediated anti-inflammatory signal by a P110δ isoform of PI3k kinase resulting in IL-10 secretion [23]. These facts suggest that MSCs are polarised into MSCs type-1 at initial stimulation of TNF-α characterised by COX2 secretion and then repolarise into MSCs type-2 along with the accumulation of inflammatory signal inside MSCs (Figure 4).

In this study, the IL-10 level tends to decrease at 10 ng/mL than 5 ng/mL dose of TNF-α. A higher dose of TNF-α cause downregulation of MSCs type-2 signalling and induce apoptotic program through TNF-related apoptosis-inducing ligand-receptor 2 (TRAIL-R2) pathway. The similar study has reported that TNF-α may induce apoptotic through TRAIL-R2 pathway [24]. TRAIL-R2 is a specific cell surface receptor belongs to the TNF receptor superfamily [25] that also expressed in MSCs [26] [27]. Upon binding TNF-α, TRAIL-R2 initiates the recruitment of Fas-associated protein with death domain (FADD) and procaspase-8 to form the death-inducing signalling complex (DISC) then activates downstream caspase-3 and leads to apoptosis [28]. These suggest that the apoptosis pathway may be activated by higher doses of TNF-α.



**Figure 4:** Schematic for MSCs polarisation under TNF-α stimulation; a) TNF-α binding to TNFR-1, then activating NF-κB and ERK signalling which produces COX2 and (b) upregulate TLR-4 expression leading to polarise MSCs into MSCs type-1 that exhibit proinflammatory profile; c) COX-2 upregulation resulting in the increase of PGE<sub>2</sub> secretion. The binding accumulation of PGE<sub>2</sub> to EP<sub>2</sub> and EP<sub>4</sub> receptors of MSCs triggering the shift of MyD88-dependent proinflammatory (MyD88-independent pathway) to TRIF-TRAM mediated anti-inflammatory signal by a P110δ isoform of the PI3k kinase, then repolarise to MSCs type 2 (anti-inflammatory phenotype) and inducing TRAF3-IRF3 resulting in IL-10 secretion. IL-10 inhibit NF-κB/IκB cytoplasmic and NF-κB nucleic then control the inflammatory milieu leading to the strong TGF-β secretion

We also found that the significant increase of TGF-β level was about 4-fold than IL-10 level (Figure 3b, P < 0.001) and shown a negative correlation between IL-10 and TGF-β production (Figure 3c).

These finding suggest that MSC rapidly respond to inflammatory milieu by polarising into immune-suppressive phenotype (MSCs type-2). The release of IL-10 has shown that the inflammation milieu is under control by MSCs, thus MSCs strongly produce other cytokine or growth factor including TGF-β production. This observation supports other studies revealing that IL-10 was elevated at 6 and 12 hour and then it decreased at 24 hour [15]. TGF-β is pleiotropic cytokines that is a well-known immunosuppressive molecule with important roles in immunoregulation [15] [19]. TGF-β inhibited inflammatory cytokine-induced iNOS expression in a SMAD3-dependent manner. In this study, we explore MSCs without the co-culture with the immune cells, thus we don't know exactly how the MSCs may interact and suppress the activated immune cells particularly in the case of an autoimmune disease.

Based on our study, we conclude that the 5 ng/mL dose of TNF-α is a sufficient dose for MSCs to suppress the inflammatory milieu. The higher increase of TGF-β is due to the controlled inflammation by IL-10.

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