

**Original article:**

**Mesenchymal Stem Cells Induce Regulatory T-cell Population in Human SLE**

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**Abstract:**

**Background:** The mechanisms underlying peripheral disorders during systemic lupus erythematosus (SLE) were found to be shared with tolerance disorders and mediated by T-regulator (T-reg) cells. Mesenchymal stem cells (MSCs) may inhibit T-cell subset differentiation and induce the T-reg cell phenotype. However, the capacity of MSCs to promote functional T-reg cells in SLE patients remains unclear. **Objectives:** This study aimed to analyze the capacity of MSCs to induce the production of functional CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T-reg cells, *in vitro*, under co-culture conditions with human SLE cells. **Methods:** This study used a pre- and post-test control group design. Peripheral blood mononuclear cells (PBMCs) were extracted from SLE patients at the Kariadi Hospital, and MSCs were derived from human umbilical cords (hUCs) The PBMC control group was treated with standard medium, and the treatment group was co-cultured with hUC-MSCs. After 24 hours of co-culture incubation, T-reg cells were removed from the PBMC pool, using magnetic-activated cell sorting (MACS), and the population was assessed using the trypan blue exclusion assay. **Results:** A significant increase in the population of T-reg cells was observed (P < 0.001) after 24 hours of co-culture incubation with hUC-MSCs. **Conclusion:** This study concluded that MSCs have the capacity to enhance the T-reg population in human SLE PBMCs.

**Keyword:** MSCs, T-regs, SLE, T-cells, PBMCs

*Bangladesh Journal of Medical Science Vol. 19 No. 04 October '20. Page : 743-748  
DOI: <https://doi.org/10.3329/bjms.v19i4.46635>*

**Introduction**

Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease, in which the body's immune system produces excessive antibodies or other soluble molecules that trigger excessive inflammatory

responses<sup>1,2</sup>. SLE patients continue to be at increased risk for premature mortality, according to a cohort study (1999-2014), indicating that mortality has not significantly improved among SLE patients, particularly among young adults<sup>3</sup>. These findings

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indicate that the development of new therapeutic agents is necessary to improve SLE management strategies. The mechanisms underlying peripheral disorders in SLE were found to be shared with those that underlie common tolerance disorders, which are mediated by CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory T (T-reg) cells. The disruption of T-reg cells may cause T- and B-cell hyperactivity, which reduces the function and number of T-reg cells<sup>4</sup>.

A previous study has reported that a decrease in circulating T-reg cells may promote the appearance of autoreactive T- and B-cells, the loss of homeostasis, and immune system failure, which is associated with SLE occurrence<sup>5</sup>. Another study reported that the enhancement of T-reg cell functions may have useful effects for human SLE<sup>6</sup>, and an SLE treatment that is currently being developed is the transplantation of mesenchymal stem cells (MSCs)<sup>7</sup>. MSCs are non-hematopoietic, plastic-adherent, multipotent, and fibroblastic-like cells that may express several surface markers, such as CD90, CD105, CD44, CD73, and lack the expression others, such as CD79, CD19, CD14, CD11b, CD45, CD34, and human leukocyte antigen (HLA)-DR<sup>8</sup>. MSCs can be differentiated into several cell and tissue types, including chondrocytes, osteoblasts, adipocytes, neural cells, and several types of immune cells<sup>9</sup>. MSCs can also inhibit the proliferation and expansion of T- and B-lymphocytes, dendritic cells, and natural killer (NK) cells. These immunosuppressive capacities have been supported by the identification of several specific mechanisms, such as the activation and expression of inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) and the enhancement of suppressive cytokines<sup>10</sup>.

Several T-cell regions are known to play roles in inflammatory regulation<sup>11,12</sup>. A previous study revealed that MSCs may inhibit the differentiation of Th17 cells and induce the T-reg phenotype<sup>13</sup>. Moreover, other studies have reported that MSCs may enhance the expansion of T-reg cells through the induction of CD4<sup>+</sup> T-cells, mediated by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1)<sup>14,15</sup>. These findings indicated that MSCs may exert anti-inflammatory effects, even under inflammatory conditions. However, the capacity of MSCs to promote functional T-reg cells in SLE patients remains unclear. The aim of our study was to analyze the capacity of MSCs to induce the production of functional CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T-reg cells, *in vitro*, under co-culture conditions with human SLE cells.

## Methods

### Research design

This study used a pre- and post-test control group design. This study was conducted at the Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia, from September–October 2018. This research used peripheral blood mononuclear cells (PBMCs), which were extracted from SLE patients at the Kariadi Hospital after obtaining informed consent, and MSCs derived from human umbilical cords (hUCs). The control group comprised PBMCs treated with standard medium, and the treatment group comprised PBMCs co-cultured with hUC-MSCs.

### MSC Isolation

This study was approved and was performed in accordance with the guidelines established by the Committee Ethics Institutional Review Board of the Medical Faculty, Sultan Agung Islamic University, Semarang, Indonesia. MSCs were isolated and separated from cord blood obtained from donors, who provided informed consent. The isolation and expansion of MSCs were performed as described previously<sup>20</sup>. Briefly, cords were cut into smaller pieces and transferred into a T25 culture flask (Corning, Tewksbury, MA, USA) containing Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Louis St, MO) and augmented with 10% fetal bovine serum (FBS, Gibco™ Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100  $\mu$ g/mL) (Gibco™ Invitrogen, NY, USA). Cultured cells then were incubated at 37°C and 5% CO<sub>2</sub>. The cell medium was renewed every 3 days, and cells were passaged after reaching 80% confluence (approximately every 14 days). hUC-MSCs from passages 4–6 were employed for this study.

### Characterization of MSCs

MSC surface antigens were analyzed by flow cytometry analysis at the fourth passage, according to company protocols. The cells were subsequently stained with allophycocyanin (APC)-mouse anti-human CD73 (Clone AD2, 560847; BD Biosciences), fluorescein isothiocyanate (FITC)-mouse anti-human CD90 (Clone 5E10, 561969 BD Biosciences), phycoerythrin (PE)-mouse anti-human CD44 (Clone G44-26, 555479; BD Biosciences), and peridinin-chlorophyll-protein (PerCP)-Cy5.5.1-mouse anti-human CD105 (Clone 266, 560819, BD Biosciences) antibodies. Cells were stained with

specific antibodies for 30 minutes, at 4°C, examined with a BD FACSAria™ II flow cytometer (BD Biosciences), and analyzed with BD FACSDiva™ software (BD Biosciences).

#### ***In vitro* differentiation assay**

MSCs were cultured to a density of  $1 \times 10^4$  cells/well in a 24-well plate in osteogenic medium, containing 50  $\mu\text{M/L}$  ascorbate-2-phosphate 10-7, mol/L 0.1  $\mu\text{M}$  dexamethasone, 10 mM/L  $\beta$ -glycerophosphate (Sigma-Aldrich, Louis St, MO), and 10% FBS in DMEM, at 37°C and 5%  $\text{CO}_2$ . After 21 days of induction, cells were washed and fixed with phosphate-buffered saline (PBS) and cold 70% ethanol (v/v), at room temperature. for 1 hour. Then, cells were washed three times with twice-distilled water. Calcium deposition was evaluated by adding 1 ml 2% Alizarin Red solution (w/v) (pH 4.1-4.3), followed by incubation at 37°C for 30 minutes, then washing four times in distilled water.

#### **Isolation of PBMCs and co-culture with MSCs**

Human PBMCs, from healthy volunteers who provided informed consent, were separated using Ficoll-Paque (Sigma-Aldrich, Louis St, MO) density-gradient centrifugation, in a 15-mL conical tube. PBMCs were cultured and expanded in 2 ml of advanced RPMI 1640 culture medium (Gibco™ Invitrogen, NY, USA), supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, and 2 mM glutamine, and incubated at 37°C, in a humidified atmosphere containing 5%  $\text{CO}_2$ . For the treatment group, PBMCs were co-cultured with MSCs in a T25 flask, in RPMI supplemented with 1% penicillin and streptomycin and 10% FBS, at a 1:40 ratio of MSCs:PBMCs, for 24 hours. For the control group, isolated PBMCs ( $1 \times 10^7$  cells/flask) were cultured in a T25 flask, with the standard medium, for 24 hours.

#### **T-reg cell sorting and counting**

After co-culture incubation, T-reg cells were isolated from the PBMC pool, using the  $\text{CD4}^+ \text{CD20}^+$  Regulatory T-Cell Isolation Kit (Miltenyi Biotec, Germany).  $\text{CD4}^+$  and  $\text{CD25}^+$  T-cells were removed from the pool by positive and negative selection, respectively. After incubation with antibodies,  $\text{CD4}^+ \text{CD25}^+$  T-reg cells were sorted, using magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Germany), according to the manufacturer's instructions. The viability and population of T-reg cells were then analyzed using the trypan blue exclusion assay and an automated cell counter.

#### **Data analysis**

All values are presented as the mean  $\pm$  standard deviation (SD). All calculations were performed using SPSS 16.0 (IBM Corp., Armonk, NY, USA). Group comparisons were analyzed by paired Student's  $t$ -tests, followed by Fisher's least-significant difference (LSD) post hoc test. A  $p$ -value of  $< 0.05$  was considered to be significant.

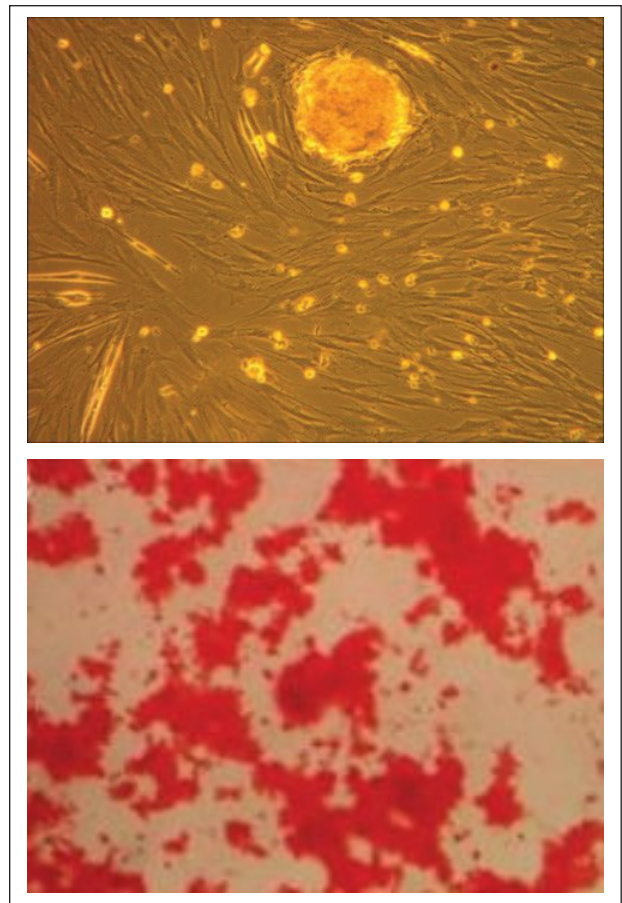
#### **Ethical Clearance**

All research activities were performed in accordance with and approved by the Health Research Ethical Committee Medical Faculty of Universitas Sumatera Utara (USU) Medan, under No. 564/TGL/KEPK FK USU-RSUP HAM/2018.

#### **Results**

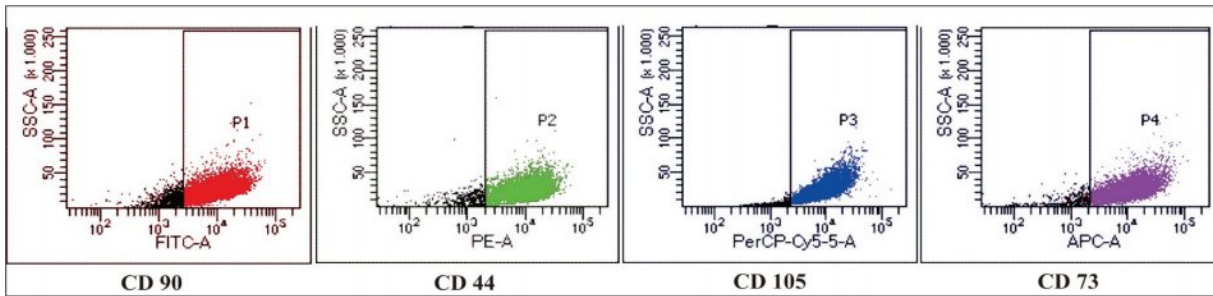
##### ***Isolation and Differentiation of hUC-MSCs***

hUC-MSCs were isolated and cultured from UCs, based on their plastic attachment capacities,



**Figure 1.** (a) hUC-MSCs were characterized by fibroblast-like appearance and spindle shape characteristics; (b) and calcium deposition appeared as a red color after Alizarin Red staining (10 $\times$  magnification).





**Figure 2.** Immunophenotyping analysis of hUC-MSCs, which were positive for CD44, CD73, CD90, and CD105.

under standard culture conditions for 4 passages. The cells were fibroblast-like, with spindle shape characteristics, became 80% confluent after 5-7 days in culture, and were regularly passaged (Figure 1a). Differentiation was examined by the administration of osteogenesis medium for 21 days. Calcium deposition was observed as a red color after immunodetection by Alizarin Red (Figure 1b).

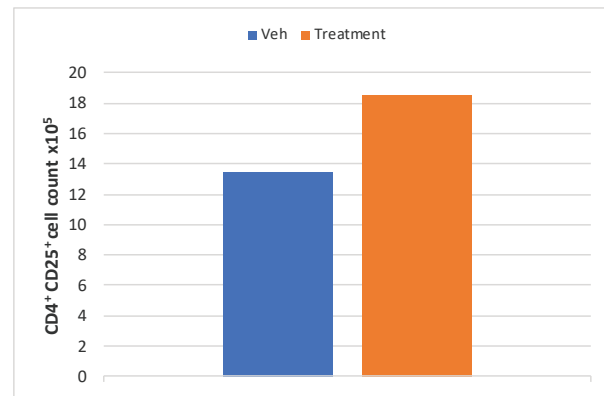
#### **Characteristics of hUC-MSCs**

hUC-MSCs were cultured for 4 passages, under standard culture conditions. After expansion, cells were passaged when they reached 80% confluence, and cell surface markers were analyzed using flow cytometry. These cells expressed high levels of CD44 (92.4%), CD73 (94.9%), CD90 (86.6%), and CD105 (96.8%), demonstrating the characteristic hMSC immunophenotype.

#### **MSCs enhance the T-reg population**

We co-cultured the hUC-MSCs with PBMCs and examined directed autoimmunity. hUC-MSCs were co-cultured with PBMCs in the treatment group, and T-reg cell generation was compared with that for the control group after 24 hours of incubation. We found that hUC-MSCs significantly increased the T-reg cell population when co-cultured with PBMCs derived from SLE patients ( $P < 0.001$ ). The number of T-reg cells among PBMCs after co-cultured with hUC-MSCs was  $18.5 \pm 1.84 \times 10^5$  cells, which was significantly higher than that for the control group ( $13.5 \pm 1.274 \times 10^5$  cells; \*\*,  $P < 0.001$ ).

We co-cultured the hUC-MSCs with PBMCs and examined directed autoimmunity. hUC-MSCs were co-cultured with PBMCs in the treatment group, and T-reg cell generation was compared with that for the control group after 24 hours of incubation. We found that hUC-MSCs significantly increased the T-reg cell population when co-cultured with PBMCs derived from SLE patients ( $P < 0.001$ ).



**Figure 3.** Regulatory T-Cell Populations after 24 hours of incubation.

#### **Discussion**

The capacity of hUC-MSCs to modulate T-cell inflammatory conditions and proliferation may represent an alternative therapeutic route for clinical use. Several studies have shown that MSCs have the capacity to suppress inflammatory niches, depending on different mechanisms, including the capacity to generate and upregulate functional T-reg cells<sup>16</sup>. Several studies have revealed that CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells, when co-cultured with hUC-MSCs, can inhibit alloantigen-activated T-cell proliferation<sup>17</sup>. Another study reported that MSCs could induce the transformation of CD4<sup>+</sup> T-cells into T-reg cells through cell contacts the contributions of PGE2 and TGF- $\beta$ 1<sup>18</sup>. MSCs may enhance the production of functional T-reg cells in human SLE patients. To prove this statement we co-cultured the hUC-MSCs with PBMCs and then sorted using MACS to find T-reg cell generation.

Our study showed that MSCs significantly increased the population of T-reg cells after 24 hours of co-culture with human SLE PBMCs, at a ratio of 1:40 MSCs to PBMCs. (Figure 3,  $P < 0.001$ ). This result suggests that MSCs may generate T-reg under SLE conditions, which have been demonstrated to occupy

an inflammatory niche with several inflammatory T-cell subsets. Under inflammatory conditions, MSCs may upregulate the expression of Toll-like receptor 3 (TLR3), leading to the release of various anti-inflammatory molecules, including interleukin (IL)-10<sup>19</sup>. The binding of several inflammatory cytokines to MSC receptors may activate nuclear factor (NF)- $\kappa$ B and extracellular signal-regulated kinase (ERK) signaling, leading to the upregulation of cyclooxygenase-2 (COX2) and TLR-4<sup>20</sup>. This condition may enhance the expression of PGE<sub>2</sub>, which binds with the prostaglandin receptors EP<sub>2</sub> and EP<sub>4</sub>, activating the TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF)-TRIF-related adaptor molecule (TRAM)- mediated anti-inflammatory signaling pathway and resulting in the expression of several anti-inflammatory molecules, such as IL-10 and TGF- $\beta$ , which may suppress the inflammatory niche<sup>21</sup>.

These regulated inflammatory conditions may lead to the generation of T-reg cells, induced by the expression of TGF- $\beta$ . A previous study revealed that TGF- $\beta$  could cause non-T-reg cells to transform into T-reg cells and inhibit effector T-cell development<sup>22</sup>. Another study revealed that TGF- $\beta$  plays a key role in signaling pathway regulation, inducing and preserving FoxP3 suppressor function and expression<sup>23</sup>.

These findings suggested that MSCs could induce T-reg cells, to modulate alloresponses into suppressive responses on effectors, resulting in the inhibition of the immune response to alloantigens. However, in our study, we did not explore TGF- $\beta$  expression or the correlation between TGF- $\beta$  and Foxp3 marker

expression in T-reg cells.

## Conclusion

Based on our study, we concluded that MSCs have the capacity to enhance the T-reg cell population in human SLE PBMCs.

## Acknowledgments

The authors gratefully acknowledge that the present research has been supported by the Ministry of Research and Technology, the Higher Education Republic of Indonesia, and Research and Community Service, Universitas Sumatera Utara (USU). The support was provided by the research grant TALENTA for 2018 [Number 2590/UN5.1.R/2018 Tanggal 16 Maret 2018]. We would also like to thank the Stem Cell and Cancer Research (SCCR) Laboratory, Medical Faculty, Sultan Agung Islamic University (Unissula), Semarang, Indonesia, for providing the necessary facilities to perform this research.

## Conflict Of Interest

The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

## Author's Contributions

Data gathering and study conception: Putra A, Ikhsan R, Darlan DM, Alif I, and Munir D

Study design: Putra A, Alif I, and Prasetyo A

Data gathering: Darlan DM, Alif I, Kustiyah AR, Ikhsan R, and Suntoko B

Writing and submitting manuscript: Putra A, Alif I, and Prasetyo A

Editing and approval of final draft: Putra A

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