ORIGINAL ARTICLE

In vitro regulation of IL-6 and TGF-ß by mesenchymal stem cells in systemic lupus erythematosus patients

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ABSTRACT

Aim To analyse the ability of mesenchymal stem cells (MSCs) to regulate interleukin 6 (IL-6) and transforming growth factor (TGF-β) expression *in vitro* under co-culture conditions in human systemic lupus erythematosus (SLE).

Method This study used a post-test group design that used peripheral blood mononuclear cells (PBMCs) from SLE patients at Kariadi Hospital, Semarang, Indonesia, and MSCs from a human umbilical cord. The cells were divided into two groups. The control group of PBMCs was treated with a standard medium, and the treatment group was co-cultured with the MSCs at a 1:40 ratio. Following 24 h incubation, the levels of IL-6 and TGF-β released in the culture medium were measured using a specific ELISA assay.

Results This study showed a significant decrease in IL-6 level ($p< 0.05$) and a significant increase in TGF-β level ($p<0.001$) following 24 h of co-culture incubation of human SLE PBMCs cells and MSCs.

Conclusion The PBMCs-to-MSCs ratio of 1:40 can regulate the IL-6 and TGF-β levels in human SLE PBMCs.

Key words: autoimmune disease, cytokine dysregulation, immunoregulation, inflammatory disorder

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by the excessive production of autoantibodies, immune complex formation, and systemic or organ-specific inflammation triggering broad inflammatory responses (1). Although SLE patient survival has improved in the past few years, excess mortality has not improved substantially (2). Mediation between the SLE cytokine tsunami and several T helper cell (Th) subsets is unbalanced, abnormalities resulting from degradation of production regulatory T cells (Tregs); this may affect the inflammatory niche (3).

Mesenchymal stem cells (MSCs) are a plastic-adherent stromal cell with a multipotent differentiation capacity (4); this may inhibit T helper cells and effector T cells, autoantibody production by B cells, and other homeostatic depletions leading to immune system failure (5). The MSCs may also fight excessive inflammatory niches by enhancing the production of Tregs. These qualities are supported by the expression of inducible nitric oxide synthase and indoleamine 2,3-dioxygenase, in addition to several anti-inflammatory cytokines, including interleukin 10 and transforming growth factor (TGF-β) (6)but the underlying mechanisms remain largely unknown. The aim of this study was to investigate how allogeneic MSCs mediate immunosuppression in lupus patients. Methods The effects of allogeneic umbilical cord-derived MSCs (UC-MSCs; these molecules may inhibit Th1, Th2, and Th17, which produce several excessive proinflammatory cytokines, including, specifically, interleukin 6 (IL-6) (6,7)but the underlying mechanisms remain largely unknown. The aim of this study was to investigate how allogeneic MSCs mediate immunosuppression in lupus patients. Methods The effects of allogeneic umbilical cord-derived MSCs (UC-MSCs.

Interleukin 6 usually mediates excessive inflammatory responses. IL-6 is a potent proinflammatory cytokine; its wide range of biological activities play an essential role in immune regulation (8). The primary sources of IL-6 are monocytes, fibroblasts, endothelial cells, B cells, and T cells. Notably, IL-6 is produced by Th2 cells with a role in the differentiation of B lymphocytes into mature plasma cells that secrete immunoglobulins (9). The overactivation of Th2 in SLE may cause IL-6 overexpression leading to enhanced autoantibody secretion (10). Abnormalities in these mechanisms, critical factors for self-tolerance and immune homeostasis maintenance, are also caused by downregulation of TGF-β, commonly produced by Tregs and known to regulate cellular functions such as proliferation, differentiation, migration, and survival, particularly by inhibiting several Tregs, such as Th1 and Th2 (11).

A previous study revealed that under sufficient levels of inflammatory niche, MSCs might impede differentiation of Th1 and Th2 and simultaneously inhibit potent inflammatory cytokines such as IL-6; this is a result of the production of Tregs through the expression of anti-inflammatory cytokines such as TGF-β (12). These findings demonstrate the possibility that MSCs impede the inflammatory niche in SLE. However, the capacity of MSCs to regulate *in-vitro* IL-6 and TGF-β production in SLE patients remains unclear.

Previously, we reported that MSCs induce the generation of Treg cell population in PBMCs of SLE patients, indicating an enhancement of the modulation of proinflammatory milieu (13).

The aim of this study was to analyse the ability of MSCs to regulate IL-6 and TGF-β expression *in vitro* under co-culture conditions in human SLE.

PATIENTS AND METHODS

Patients and study design

This post-test control group study was conducted in the Stem Cell and Cancer Research Laboratory at the Sultan Agung Islamic University's Faculty of Medicine in Semarang, Indonesia, between September and October 2018. The study used peripheral blood mononuclear cells (PBMCs) of three SLE patients from Kariadi Hospital, Semarang, Indonesia, and MSCs from the last third of a donor umbilical cord. The study samples were divided into two groups (three replications from three patients); the control group was treated with a standard medium, and the treatment group received a treatment co-cultured with hUC-MSC.

An informed consent was received from all patients' legal guardians after providing beneficial information prior to the study.

This study was approved by the Ethics Committee of the institutional review board of Sultan Agung Islamic University's Faculty of Medicine, Semarang.

Methods

Mesenchymal stem cell isolation. The MSCs were isolated from an umbilical cord obtained from a donor. The umbilical cord was cut into smaller pieces and transferred to a T25 culture flask (Corning, Tewksbury, MA, USA) containing Dulbecco's modified eagle medium (Sigma-Aldrich, Louis St, MO), supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco, Invitrogen, NY, USA). Umbilical cord tissue was then incubated at 37 °C in a humid atmosphere consisting of 5% CO_2. The medium was renewed every three days, and the cells were passaged after reaching 80% confluence (14 days). The following experiments used hUC-MSCs at passage fourth.

Characterization of MSCs. Surface antigens akin to MSCs were analysed by flow cytometry analysis at the fourth passage according to company protocols. The cells were subsequently incubated in the dark with allophycocyanin mouse anti-human CD73 antibodies, fluorescein isothiocyanate mouse anti-human CD90 antibodies, perCP-Cy5.5.1 mouse anti-human CD105 antibodies, and phycoerythrin mouse anti-human CD45/CD34/CD11b/CD19/HLA-DR-negative Lin antibodies. According to the International Society of Cellular Therapy (14), the specific marker profile expressed by MSCs comprises CD73, CD105, and CD90, and CD45/CD34/CD11b/CD19/HLA-DR negative Lin.

The analysis was performed using BD Stemflow (BD Biosciences, San Jose, CA, USA). The antigens were stained with a specific antibody for 30 minutes at 4 °C, examined with a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed using BD Accuri C6 Plus software (BD Biosciences, San Jose, CA, USA.

In-vitro osteogenic differentiation assay. To characterize the differentiation capacity of MSCs, the cells were cultured at a density of 1.5 x 10^4 cells per well. The cells were grown at 37 °C, 5% CO_2, and ≥95% humidity in 24-well plates featuring a standard medium comprising Dulbecco's modified eagle medium (Sigma-Aldrich, Louis St, MO) supplemented with 10% FBS (Gibco Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco Invitrogen, NY, USA). Upon reaching 95% confluence, the standard medium was aspirated and replaced with an osteogenic differentiation medium containing Human MesenCult Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), augmented with 20% Human MesenCult Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore), and 1% L-Glutamine (Gibco, Invitrogen, NY, USA). Differentiation mediums were renewed every three days. Upon bone matrix formation, osteogenic differentiation was visualized by staining with 1 ml of 2% alizarin red solution.

Isolation of PBMCs and MSCs co-culture. Human PBMCs were separated using a Ficoll-Paque (Sigma-Aldrich, Louis St, MO) density gradient centrifugation in a 15 mL conical tube from three SLE patient volunteers who gave specific, informed consent. The 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, 2 mM glutamine and incubated at 37 °C in a humidified atmosphere with 5% CO_2. For the treatment group, following 24 h incubation, PBMCs were co-cultured with MSCs on 24-well plates cultured in RPMI supplemented with 1% penicillin-streptomycin and 10% FBS at an MSCs-to-PBMCs ratio of 1:40 for 24 h, in accordance with the aforementioned previous study (12). For the control group, following 24 h incubation, the isolated PBMCs were co-cultured with the standard medium in another 24-well-plate for 24 h.

ELISA assay. A specific ELISA assay measured the levels of IL-6 and TGF-β released in the culture mediums containing cells from both the treatment and control groups. The levels of IL-6 and TGF-β were measured according to the manufacturer's instructions (Fine Test, Wuhan, China). Colorimetric absorbance was recorded at a wavelength of 450 nm.

Statistical analysis

Group comparisons were made using a paired *t*-test followed by Fisher's Least Significant Difference test. A $p<0.05$ was considered significant.

RESULTS

Isolation and differentiation of MSCs

Umbilical-cord-derived MSC-like cells were successfully isolated based on their plastic adherent capability under standard culture conditions. After passage four, MSCs had a stable fibroblast-like morphology with spindle shape (Figure 1A). MSCs showed their multipotent characteristic through differentiating into osteoblast under inductive culture conditions (Figure 1 B).

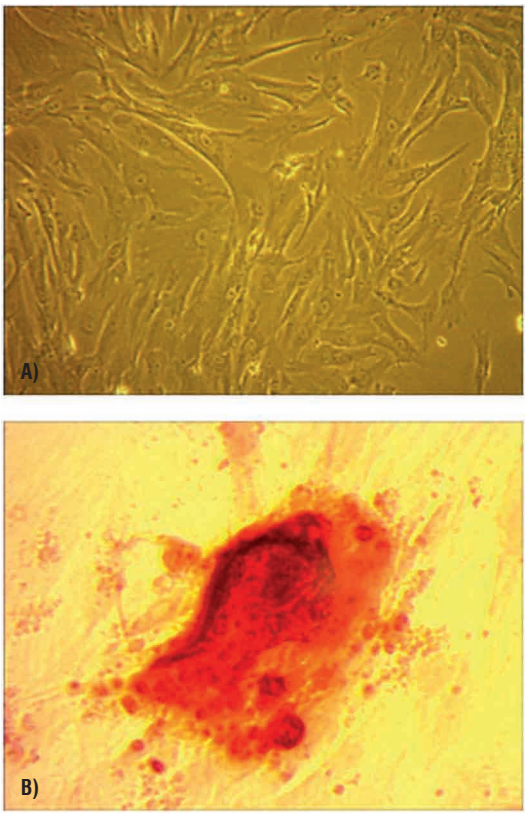

Figure 1. A) Umbilical cord mesenchymal stem cells characterized by being fibroblast-like with a spindle shape; B) calcium deposition shown in red by red alizarin staining (400x magnification)

MSCs were also positive for CD73, CD90 and CD105 and negative for the hematopoietic lineage markers (CD45, CD34, CD11b, CD19, HLA-DR) (Figure 2).

The results showed that IL-6 level was significantly decreased *(*$p<0.05$) in the treatment group (639.35 ± 96.33 pg/mL) following 24 h incubation of the MSCs. However, a significant increase ($p< 0.05$) in TGF-β levels (150.80 ± 7.06 pg/mL) was observed (Figure 3).

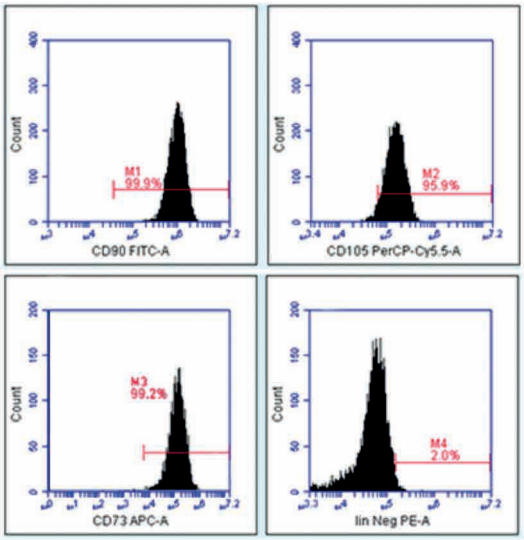

Figure 2. Immunophenotyping analysis of mesenchymal stem cells, which was positive for CD73, CD90, and CD105 antibodies and negative for Lin antibodies

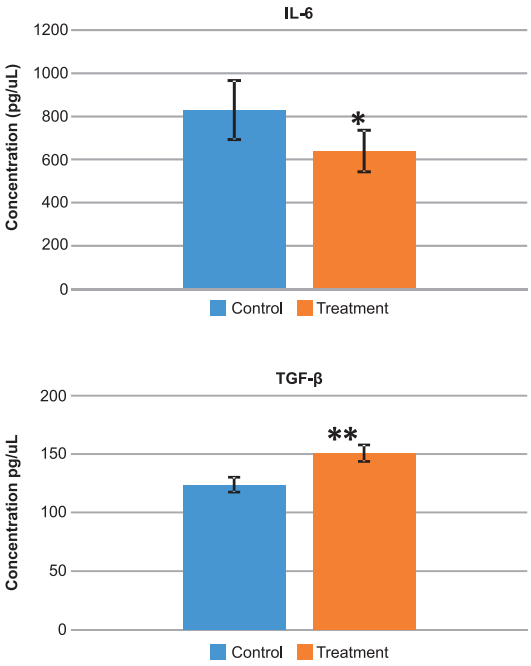

Figure 3. ELISA assays showed a significant decrease of interleukin 6 levels in the treatment group (639.35 ± 96.33 pg/mL) (left); a significant decrease was observed in transforming growth factor level (150.80 ±7.06 pg/mL) (right)

DISCUSSION

The ability of MSCs to regulate Treg proliferation has promoted alternative clinical therapies (13). However, though several studies have revealed that MSCs can suppress the inflammatory niche through different mechanisms (15,16), the underlying mechanisms of MSCs-mediated immunoregulation in SLE patients remained incompletely understood. This study has demon-

strated that hUC-MSCs regulate the inflammatory niche in SLE patients by downregulating IL-6 levels and upregulating TGF-β levels.

There are several immune tolerance breakdowns in SLE; these involve autoantigens and are present in several cell types, making antigen-specific therapy design difficult (17). Current SLE therapies inhibiting both T and B lymphocytes carry the risk of adverse events, such as infection (7,17)these cells also display multiple potent immunomodulatory capabilities, including allosuppression, making allogeneic cell therapy a possibility. The exact mechanisms involved in regulatory T cell induction by allogeneic human MSC was examined, using purified CD4+ populations and well-characterized bone marrow-derived adult human MSC. Allogeneic MSC were shown to induce forkhead box P3 (FoxP3. The unique immunoregulatory capacities of MSCs include impeding T cell proliferation and inducing Treg generation, inhibiting B cell function and immunoglobulin production, and regulating the excessive proinflammatory niche, particularly in the context of human SLE (19). A previous study revealed that, under inflammatory conditions, MSCs might simultaneously inhibit Th1 and Th2 differentiation and decrease several potent inflammatory cytokines through the production of Tregs activated by TGF-β (20)mesenchymal stromal cells (MSCs.

This study demonstrated that MSCs might regulate the inflammatory niche by inhibiting the overexpression of potent proinflammatory cytokines and simultaneously enhancing the anti-inflammatory mechanism in human SLE. The study showed that at an MSCs-to-PBMCs ratio of 1:40 in a 24 h co-culture MSCs might inhibit IL-6 expression in human SLE PBMCs. These data suggest that MSCs may impede the inflammatory niche under SLE conditions. At a sufficient proinflammation level MSCs may upregulate the expression of TLR-3, leading to the release of various anti-inflammatory molecules, including interleukin 10 (21). The NF-κβ and ERK pathways activated in MSCs by several inflammatory cytokines binding may lead to cyclooxygenase-2 and TLR-4 upregulation (22). Upregulation of PGE_2 may lead those molecules to bind to EP2 and EP4 receptors, activating TRIF-TRAM mediated anti-inflammatory signals and resulting in anti-inflammatory expressions such as interleukin 10 and TGF-β (23).

Accordingly, our study demonstrated a significant increase in TGF-β levels in human SLE PBMCs, following co-culturing at an MSCs-to-PBMCs ratio of 1:40 for 24 h. In inflammatory diseases, MSCs may express TGF-β, which may regulate multifaceted cellular functions, including inhibiting several effectors and Th subsets. The previous study demonstrated that TGF-β could promote Tregs and inhibit effector T cell development (24). Another study demonstrated TGF-β's critical role in regulating the signaling pathway that commences and preserves the FoxP3 suppressor function (25)Th2, or T regulatory (Treg. However, TGF-β expressed by Tregs occupy an autocrine position, excessively expressing anti-inflammatory cytokines and regulating several inflammatory-mediated several Th subsets, including, specifically, Th2. Inhibited Th2 development is caused by TGF-β expression; this may inhibit the GATA-3 transcription factor (26) increased levels of cytokines and their receptors can be observed in target organs, and it is clear that they have important roles in disease pathogenesis. Recent therapeutic strategies have focused on proximal cytokines, such as interferon-α, interleukin (IL. Thus, degradation of Th2 generation also results in the downregulation of IL-6 molecules as potent proinflammatory cytokines (27). These findings suggest that MSCs could regulate inflammatory conditions in human SLE by downregulating IL-6 and upregulating TGF-β expression. However, this study did not explore other potent cytokines, related transcription factors, or the generation of different Th cell subsets.

In conclusion, we have shown here that MSCs could hamper the IL-6 level and enhance the TGF-β level in human SLE PBMCs at a 1:40 ratio of MSCs to PBMCs. This finding is important for understanding the capability of MSCs as a potential immunomodulator, particularly in SLE.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.