ORIGINAL ARTICLE

Revealing the decrease of indoleamine 2,3-dioxygenase as a major constituent for B cells survival post-mesenchymal stem cells co-cultured with peripheral blood mononuclear cell (PBMC) of systemic lupus erythematosus (SLE) patients

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ABSTRACT

Aim Mesenchymal stem cells (MSCs) have potent immunosuppressive properties to control systemic lupus erythematosus (SLE) disease by inhibiting indoleamine 2,3-dioxygenase (IDO), and increasing regulatory T cells (Treg) to control innate and adaptive immune cells. However, the interaction and mechanism regarding IDO and B cells in the co-culture of MSC and SLE peripheral blood mononuclear cell (PBMCs) remain unclear. This study aimed to investigate the effects of MSCs in controlling B cells through IDO expression in PBMC of SLE patients.

Methods This study used a post-test control group design. MSCs were obtained from human umbilical cord blood and characterized according to their surface antigen expression and multilineage differentiation capacities. PBMCs isolated from SLE patients were divided into five groups: sham, control, and three treatment groups. The treatment groups were treated by co-culturing MSCs to PBMCs with a ratio of 1:10, 1:25, and 1:40 for 72 h incubation. The B cell levels were analysed by flow cytometry with cytometric bead array (CBA) and the IDO levels were determined by ELISA.

Results The percentages of B cells decreased significantly in groups treated by dose-dependent MSCs, particularly in T1 and T2 groups. These findings were aligned with the significant decrease of the IDO level.

Conclusion MSCs control B cells-mediated by a decrease of IDO in PBMC of SLE patients.

Key words: autoimmune disease, immunoregulation, inflammatory disorder

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a highly complex multisystem disease characterized by dysfunction of lymphocyte T cells and excessive autoantibody produced by autoreactive polyclonal B cells leading to multi-organ manifestations (1). Although recent evidence reported high improvement of survival rates in the past few years, the mortality rates have still not substantially improved (2). The traditional therapy of SLE including immunosuppressive drugs have multiple side effects and remain temporarily effective to modulate proinflammatory milieu in flaring SLE conditions (3). Hence, finding the potent and effective alternative therapies with minimum side effects and enduring immunomodulatory effects are a crucial need. Recently, the use of mesenchymal stem cells (MSCs) has emerged as a new therapeutic option in SLE due to their immunomodulatory properties to suppress the massive proinflammatory immune cells, including T and B lymphocytes (4). A previous study reported that, as a major enzyme in tryptophan catabolism, indoleamine 2,3-dioxygenase (IDO) has a critical role in controlling the proliferation of autoreacted B cells (5). However, the modulatory effect of MSCs to control IDO-induced B cells in SLE is still unclear. Therefore, revealing the interaction between B cells survival and IDO level following MSC administration in SLE is needed.

MSCs are a self-renewing cell population with fibroblast-like characteristic, which have capability to differentiate into a wide variety of tissues, including adipocytes, chondrocytes, osteocytes and neurons. These multipotent cells also have a plastic-adherent capability and must express several surface antigens, including CD73, CD90 and CD105 with a lack of hematopoietic lineage markers such as CD11b or CD14, CD19 or CD79, CD34, CD45 and HLA-DR (6,7). Currently, the immunomodulatory capabilities of MSCs have been mostly revealed through expressing the potent immunosuppressive molecules to inhibit the overactivated immune effector cells in excessive inflammatory milieu (8). Because of these immunomodulatory properties to most immune cells, including B lymphocytes, MSCs extend a promising alternative treatment to excessive autoimmune disease, including SLE (9).

Recently, most studies reported that MSCs could preserve as immunomodulatory agent to hamper the activation and proliferation of effector lymphocytes, including B cells through releasing several potent anti-inflammatory molecules, such as IL-10, TGF-β, PGE2 and IDO. IDO, an interferon-γ (IFN-γ)-inducible intracellular enzyme, catalyzes the first step in tryptophan degradation that is expressed by professional antigen-presenting cells (APCs) to exert important immunosuppressive functions in controlling the inflammatory milieu by inhibiting B cell maturation (10). A previous study also reported that MSCs might suppress immunoglobulin production by inhibiting B cell differentiation into plasma cells (PCs) and plasma dendritic cells (pDC)-induced B cell maturation under inflammation states (11). On the other hand, the differentiation of B subsets occurs mainly in response to the T-dependent antigen manner that is influenced by pro-inflammatory cytokines such as type I interferon (IFN) and IL-6 under the extra-follicular or germinal centre (GC) pathways (9). Therefore, it was proposed that the effect of MSCs on B cells is mainly governed by T cells-dependent response rather than direct effects on B cells only.

The aim of this study was to investigate the effects of MSCs in controlling B cells through IDO expression in PBMC of SLE patients.

PATIENTS AND METHODS

Patients and study design

This study was conducted in the Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia, in September-October 2019. In total, 20 SLE patients from Kariadi Hospital and 5 healthy subjects were included in the study. A specific informed consent was obtained from each subject for peripheral blood and umbilical cords (UC) collection.

The participants were divided into 5 groups: sham (Sh) and control (C) groups were treated with standard medium, and the treatment groups (T1, T2 and T3) were co-cultured with hUC-MSC at doses of 1:1, 1:25, and 1:50, respectively (MSCs:PBMCs) (12).

This study was approved by and conducted in accordance with the review board of the Health

Research Ethical Committee Medical Faculty of Universitas Sumatera Utara (USU) Medan, (No. 698/TGL/KEPK FK USU-RSUP HAM/2019).

Methods

MSCs isolation, characterization and differentiation assay. MSCs were isolated from fresh Wharton jelly of the UC obtained from healthy mothers in the local maternity hospital with specific informed consent. The isolation and expansion process was carried out as described previously (13). Briefly, the cords were rinsed using phosphate-buffered saline (PBS) (Gibco Invitrogen, NY, USA), and cord blood was removed. The washed cords were cut into smaller pieces, transferred to a T25 culture flask (Corning, Tewksbury, MA, USA) containing Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Louis St, MO) supplemented with 10% foetal bovine serum (FBS), 1% penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.25% amphotericin B (Gibco Invitrogen, NY, USA) and incubated at 37 °C in a humid atmosphere consisting of 5% CO2. The medium was renewed every 3 days. Non-adherent cells were removed by washing. After fibroblast-like cells appeared and reached 80% confluence (14 days), the cells were trypsinized and transferred into a new flask for further expansion. The cells derived from passage 5 were used for the following experiments.

MSC-like surface markers were assessed by flow cytometry analysis at passage 5 according to the manufacturer's instructions. MSC-like cells were trypsinized and pelleted by centrifugation at 1900 rpm for 8 minutes. The supernatant was removed, and pelleted cells were washed using PBS. Approximately $1x10^5$ detached cells were resuspended in 100 µL staining buffer (BD Bioscience, San Jose, CA, USA). For the staining of surface antigens, the cells were subsequently incubated using fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)-, peridinin-chlorophyll-protein (perCP)-CyTM5.5.1, and phycoerythrin (PE)-conjugated anti-human CD90, CD73, CD105, and Lin (CD45/CD34/CD11b/CD19/HLA-DR) antibodies (BD Bioscience, San Jose, CA, USA) for 30 minutes at room temperature in the dark. In addition, an appropriate isotype-specific conjugated anti-IgG (BD Bioscience, San Jose, CA, USA) was used as the negative control. Unstained cells were also used to determine a threshold. Each sample was washed twice using PBS, and the analysis was performed using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, CA, USA). A minimum of $1x10^4$ gated events on forward scatter and side scatter were recorded for each sample. Finally, post-acquisition analysis was conducted using BD Accuri C6 Plus software (BD Bioscience, San Jose, CA, USA).

The MSC-like cells were cultured at a density of 1x104 cells/well in a 24-well plate with standard medium containing DMEM, 10% FBS, 1% penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.25% amphotericin B. The cells were incubated at 37°C, 5% CO_2, and ≥ 95% humidity. After 95% confluency was reached, the standard medium was replaced with an osteogenic differentiation medium containing Human MesenCult Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), supplemented with 20% Human MesenCult Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore) and 1% L-glutamine (Gibco Invitrogen, NY, USA). The differentiation medium was renewed every 3 days. After bone matrix formation occurred, osteogenic differentiation was visualized by adding 1 ml of 2% alizarin red solution (w/v) (pH 4.1-4.3). The cells were incubated at 37°C for 30 minutes, then washed four times in distilled water.

Isolation of PBMCs and MSCs co-culture. Human peripheral blood mononuclear cells (PBMCs) were isolated from 20 active SLE patients and 5 healthy controls at the same point using Ficoll-Paque (Sigma-Aldrich, Louis St, MO) density gradient centrifugation. The separated buffy coat layer containing PBMCs was collected, washed, and pelleted by centrifugation at 1900 rpm for 8 minutes. Then, PBMCs were cultured 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 with 5% CO_2. After 24 hours of incubation, for the treatment groups, PBMCs were co-cultured with MSCs using a Corning Costar 0.4 µm Transwell cell culture plate in RPMI supplemented with 10% FBS, 1% penicillin–streptomycin, and 0.25% amphotericin B at an MSC to PBMC ratio of 1:1, 1:25, and 1:50 (T1, T2, and T3, respectively) for 72 hours. On the other hand, for the Sh and C

groups, the isolated PBMCs from healthy and SLE patients, respectively, were cultured in another well plate with standard medium for 72 hours (12).

Flow cytometry analysis. According to the manufacturer's instructions, Treg and B cell surface markers in the PBMCs population were assessed by flow cytometry analysis after 72 hours of incubation of MSCs. The PBMCs were pelleted by centrifugation at 1900 rpm for 8 minutes. The supernatant was removed, and pelleted cells were washed using PBS. Approximately $1x10^5$ detached cells were resuspended in 100 µL staining buffer (BD Bioscience, San Jose, CA, USA). For the staining of B cell surface antigens, the cells were subsequently incubated using perCP- conjugated anti-human CD19 (BD Bioscience, San Jose, CA, USA). These cells were incubated for 30 minutes at room temperature in the dark. In addition, unstained cells were also used to determine a threshold. Each sample was washed twice using PBS, and the analysis was performed using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, CA, USA). A minimum of 1x104 gated events on forward scatter and side scatter were recorded for each sample. The post-acquisition analysis was conducted using the BD Accuri C6 Plus software (BD Bioscience, San Jose, CA, USA).

Determination of IDO level. The co-culture supernatant was collected after 72 hours of incubation. IDO, IFNγ, IL-6 and IL-10 levels were determined in co-culture supernatant after 72 h incubation using specific ELISA. Briefly, according to manufacturer protocol, the IDO level was analysed using a standard curve constructed for the specific assay. The absorbance was determined at the wavelength of 450 nm.

Statistical analysis

Values were presented as the mean±SD. Group comparisons were analysed by paired ANOVA and then followed by post hoc Fisher's LSD. A $p < 0.05$ was considered significant.

RESULTS

MSCs isolated from the umbilical cord were analysed based on their plastic adherent capability under standard culture condition, antigen-specific surface markers, and differentiation capability after 5 passages. The cell morphology of MSCs at the fourth passage exhibited typical monolayers of spindle-shaped fibroblast-like cells, with adhering capability to the plastic flask (Figure 1A). To characterize MSCs surface antigens, we performed flow cytometry analysis as indicated by the International Society for Cellular Therapy (ISCT) (14). We found a high level of CD90 (99.7±2.3%), CD105 (95.1±1.5%), CD73 (99.4±1.7%), and low level of CD34, CD45, CD11b, CD19 and HLA-DR, represented as Lin (0.2±0.05%) (Figure 1C).

The ability of MSC to differentiate into osteogenic cells was analysed by culturing the MSCs under an osteogenic medium for 20 days in which the calcium deposition was visualized as red colour after alizarin red solution administration.

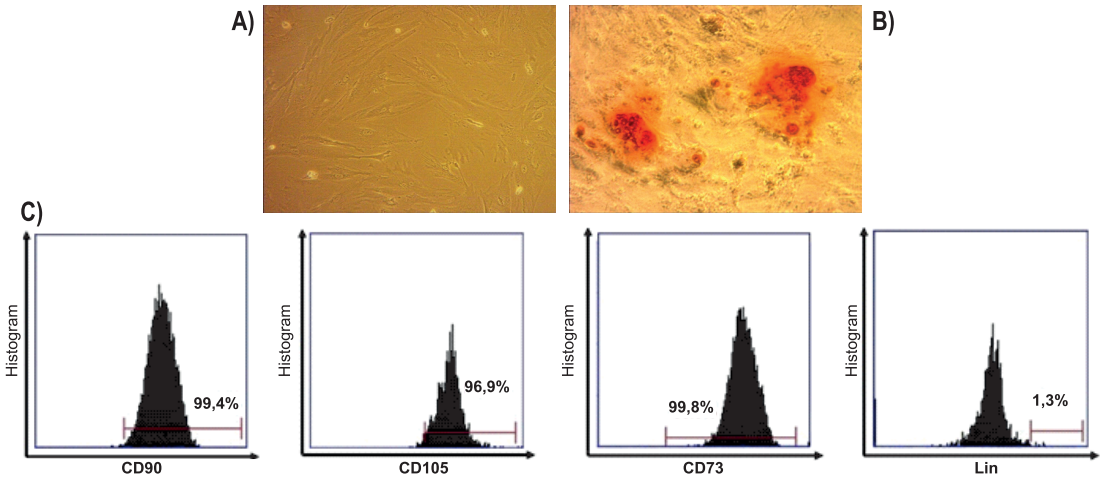

Figure 1. A) Umbilical cord-mesenchymal stem cells (UC-MSCs) candidate from the in vitro culture showed spindle form such as fibroblast-like cells (100x magnification); B) UC-MSCs were treated using an osteogenic differentiation medium to assess the capacity of MSCs to differentiate into the bone matrix. The calcium deposition appeared in red colour after alizarin red staining (200x magnification); C) Flow cytometry characterization of UC-MSCs expressed CD90, CD105, CD73, and negatively expressed CD34, CD45, CD11b, CD19, and human leukocyte antigen – DR isotype (HLA-DR)

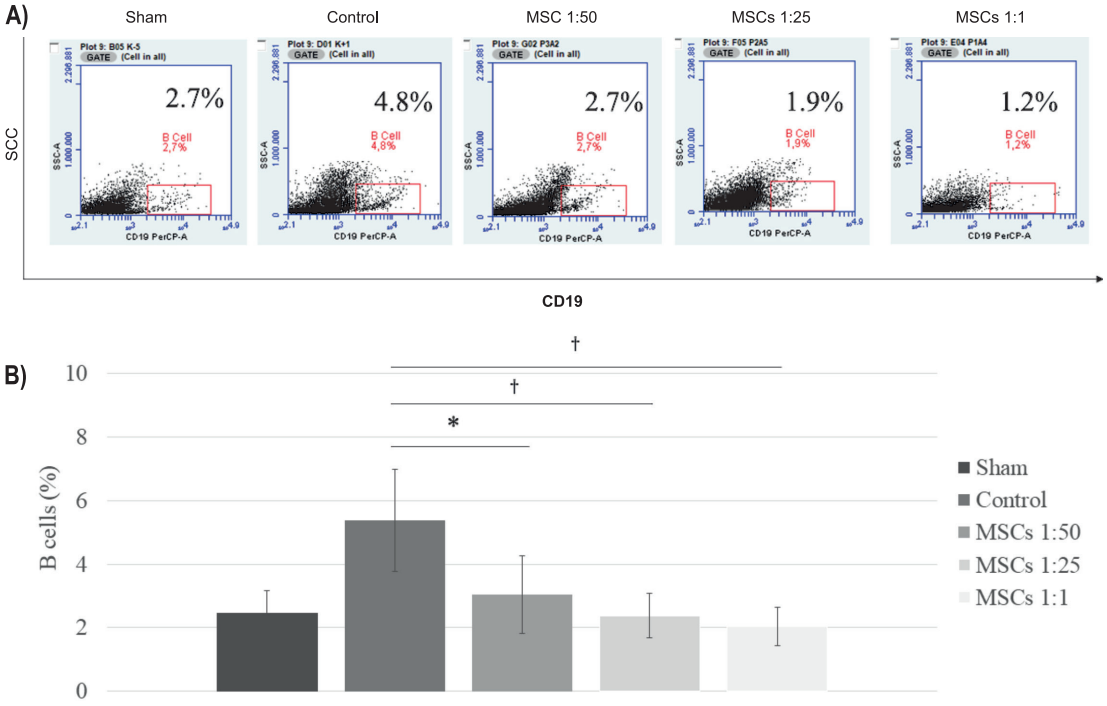

Figure 2. A) Mesenchymal stem cells (MSCs) inhibit B cell generation in systemic lupus erythematosus peripheral blood mononuclear cells (SLE PBMCs). Flow cytometry was used to identify B cells; B) Quantification of B cells was repeated three times; $p<0.05$; †$p<0.001$; (SSC: side scatter);

To confirm the *in vitro* differentiation potential of MSCs, we used an osteogenic differentiation assay to demonstrate the ability of MSCs to differentiate into osteogenic cells. Under these assays, we found a red colour in most cell cultures as calcium deposition that indicated these MSCs have differentiated into osteogenic (Figure 1B).

To investigate the capacity of MSCs in inhibiting B cells in vitro, we co-cultured MSCs with PBMCs from SLE patients for 72 h incubation and analysed the CD19 as one of the novel markers in B cells using flow cytometry (Figure 2A). This study showed that MSCs could inhibit the percentage of B cells population in which there was a significant decrease of CD19 population in the PBMCs population in the T1 and T2 groups ($p < 0.05$), in which the percentage of CD19 in T1 was 1.70±0.15 %, and in T2 was 1.9±0.59 % (Figure 2B).

To investigate the capacity of MSCs in enhancing the level of IDO, the ELISA assay was performed after 72 of hours incubation of MSCs and PBMCs

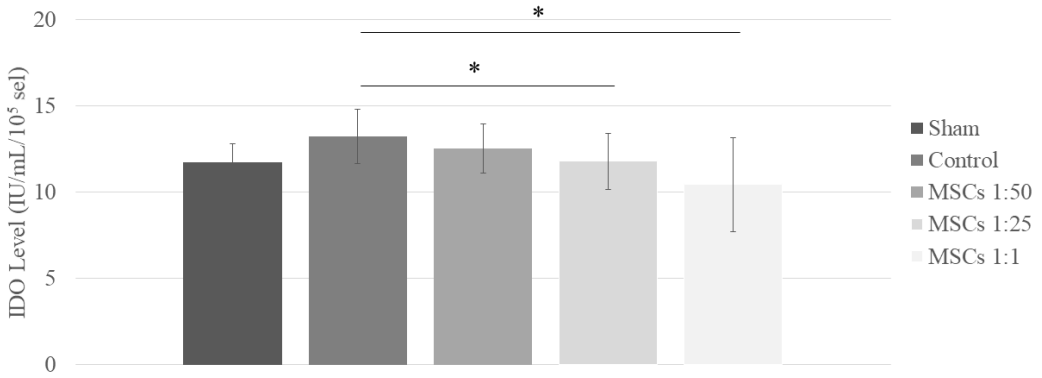

Figure 3. Mesenchymal stem cells (MSCs) decrease indoleamine 2-3 deoxygenase (IDO) level in the co-culture between MSCs and systemic lupus erythematosus peripheral blood mononuclear cells (SLE PBMCs) *in vitro*; $p<0.05$

co-culture. The ELISA assay showed that there was a significant decrease of IDO level in the T1 and T2 groups ($p < 0.05$), compared with the control group ($p<0.001$ and $p<0.05$, respectively). The level of IDO in T1 was 10.45±2.73 IU/mL, and in T2 was 11.79±1.64 IU/mL (Figure 2B, Figure 3).

DISCUSSION

This study demonstrated that MSCs significantly suppress the CD19 B cells in SLE patients by decreasing IDO. These effects were dose-dependent and an increase in the ratio of the doses of MSCs to PBMCs. The ratio of MSCs:PBMCs (1:1) decreases B cell population up to 2%. These findings were in line with previous studies that reported that MSCs suppress LPS-stimulated B cell populations (9). Our previous study also reported that MSCs suppress B cells in SLE correlate with an increase of Treg levels. Treg can inhibit B cells when the Treg was co-cultured Treg and $CD19^{+}CD27^{-}IgD^{-}$ B cells or autologous $CD19^{+}CD27^{hi}$ B cells, as the cells producing the serologic abnormalities in SLE patients with active lupus (11,15). The decrease in autoreactive B cells preventing the overproduction of autoantibodies associated with SLE improvement (16)present antigens to T lymphocytes and regulate immune responses. However, because of the inherent randomness in the process of generating their vast repertoire of antigen-specific receptors, B cells can also cause diseases through recognizing and reacting to self. Therefore, B lymphocyte selection and responses require tight regulation at multiple levels and at all stages of their development and activation to avoid diseases. Indeed, newly generated B lymphocytes undergo rigorous tolerance mechanisms in the bone marrow and, subsequently, in the periphery after their migration. Furthermore, activation of mature B cells is regulated through controlled expression of co-stimulatory receptors and intracellular signalling thresholds. All these regulatory events determine whether and how B lymphocytes respond to antigens, by undergoing apoptosis or proliferation. However, defects that alter regulated co-stimulatory receptor expression or intracellular signalling thresholds can lead to diseases. For example, autoimmune diseases can result from altered regulation of B cell responses leading to the emergence of high-affinity autoreactive B cells, autoantibody production and tissue damage. The exact cause(s.

Interestingly, this study found the decrease of IDO in line with the ratio of the doses of MSCs. IDO enzymatically degrades tryptophan resulting in an accumulation of downstream breakdown products of kynurenine to exhibit biological activity in the immune system, including by inducing Treg function via as a signalling protein shapes the immunological microenvironment (17). The downregulation of IDO following exposure to MSCs in several ratios establishes a correlation between IDO level and B cell inhibition. These effects were dose-dependent, and a decrease in the ratio of the doses of MSCs to PBMCs resulted in a 10 IU/ml/10^5 cells decrease in IDO. Decreased IDO production due to the inactivation of $CD8^{+}$ T cells which decreased the production of IFN- γ are associated with suppressing B cells population (18). The medium and high doses of MSCs reduce inflammatory mediators associated with dendritic cells, monocytes, and macrophages inactivation. These findings in line with the previous study, demonstrated that MSCs are initially unable to reduce IDO, however exposure to pro-inflammatory mediators including IFN- γ, TNFα, and IL-1 significantly decrease IDO level (19). The decreased IDO levels after MSCs administration are associated with suppressing of CD11c dendritic cells and decrease pro-inflammatory mediators such as IFN- γ, TNFα, and IL-6 (11,19,20).

In conclusion, MSCs could control B cells by decreasing of IDO level in PBMC of SLE patients under controlled B cells following MSC treatment. Therefore, the MSCs treatment may provide a promising alternative therapy to control the aberrant immune response in SLE disease.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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