










IL-6 and IL-8 Suppression by Bacteria-adhered Mesenchymal Stem Cells Co-cultured with PBMCs under TNF- α Exposure

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Abstract: The potential of mesenchymal stem cells (MSCs) in controlling bacterial infections is an evolving field to investigate. In terms of response to inflammatory cytokines, MSCs can polarize into MSCs type-1 and MSCs type-2 to reach the homeostasis process, including regulating IL-8 and IL-6. MSCs are also exhibit antimicrobial properties and regulate immune responses. This study was designed to explore the ability of MSCs to control the inflammation produced by *Staphylococcus aureus*-contaminated PBMC with TNF- α stimulation by analyzing IL-6 and IL-8 levels. We used a post-test group design with 2 study groups, consist of vehicle control (Veh) and a treatment (1:20 comparison of MSCs: PBMCs) in triplicate supplemented with *S. aureus* under 10 ng/mL TNF- α recombinant. The medium supernatant was collected after 0, 4, 8, and 12, the IL-6 and IL-8 were measured using ELISA assay. This study showed a significant increase in IL-6 and IL-8 at the first hour's incubation. Interestingly, the IL-6 and IL-8 levels were significantly decreased after 12 and 8 to 12 hours of incubation, respectively. Based on our study, we conclude that MSCs may regulate the IL-6 and IL-8 production on bacteria-contaminated PBMC with inflammation in early incubation to late incubation.


1 INTRODUCTION


Recently, mesenchymal stem cell (MSC) therapy has gained more attention in controlling the massive inflammation than other stem cells. These cells have immunomodulatory, anti-inflammatory, and


antibacterial properties in addition to differentiation capabilities (1,2). Sepsis is a systemic inflammatory response to infection, characterized by the excessive release of pro-inflammatory cytokines and disordered fibrinolysis produced by immune cells and damaged tissue (3). Although several approaches aim to reduce


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mortality rates in severe sepsis, such as goal-oriented treatments, appropriate antibiotic treatment, and corticosteroid treatment, there is still no effective treatment for the sepsis (4). The uncontrolled activation of the immune response in sepsis leads the macrophages and endothelial and epithelial cells to produce the release of cytokine cascades such as tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1, IL-6, IL-8, IL-12, IL-18, and interferon (IFN)- γ (5). However, the pro-inflammatory cytokines IL-8, the chemoattractant of neutrophil and IL-6, the induction of the acute-phase response have been studied extensively concerning its possible role in the pathogenesis of sepsis (6). Therefore, investigating the role of MSCs to control IL-8 and IL-6 under the inflammation microenvironment is currently one of the most promising options.

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent, and plastic adherent fibroblast-like cells capable of differentiation into mesenchymal and nonmesenchymal lineages (7). They are characterized by the expressions of surface markers CD73, CD90, CD105, and the absence of CD45, CD34, CD14 or CD11b, CD79a or CD19, and Human Leucocyte Antigen (HLA) class II (8,9) MSCs can differentiate into osteocytes, chondrocytes, and adipocytes under standard in-vitro differentiating conditions (7). They also can be isolated from bone marrow, mobilized peripheral blood, cord blood, umbilical cord (UC), placenta, adipose tissue, dental pulp, and even fetal livers and lungs (10). MSCs can regulate immune responses in various disease models by polarizing into MSCs type-1 as proinflammation phenotype and MSCs type-2, anti-inflammatory cells depend on inflammation exposure. MSCs can also regulate immune responses in a variety of disease models through polarizing into type-1 (proinflammation) and type-2 (anti-inflammation), depending on inflammation exposure (11). MSCs originating from either bone marrow or adipose tissue are beneficial in sepsis, indicating that MSC may upregulate antimicrobial activity in the presence of infection by releasing Antimicrobial Peptides (AMPs) (12-14).

MSCs have immunoregulator properties that can control inflammatory cells by releasing anti-inflammatory cytokine IL-10 leading to the decrease of pro-inflammatory cytokines including IL-8 and IL-6 (1,15,16). Theoretically, IL-8 belongs to the class of pro-inflammatory chemokines produced by the active macrophages post an infectious process in which its level follows a course of time similar to that of IL-6 (17). In line with IL-8, increased IL-6 also shows in response to severe infection (15). Thus, the

IL-8 and IL-6 indicated as an early marker of sepsis. However, several studies reported that MSCs could suppress severe inflammation, leading to improved sepsis and decreased sepsis animal models (2,18). Thus far, the treatment of MSCs in patients with sepsis has not been used. Furthermore, the role of MSCs to control the level of IL-6 and IL-8 released by inflammatory cells following bacteria contamination and under TNF- α stimulation in human PBMCs culture to mimic sepsis conditions remains unclear. Therefore, in the present study, we explored the ability of *Staphylococcus aureus*-adhered MSCs co-cultured with PBMCs at a comparison of 1:20 (MSCs and PBMCs) under 10 ng/mL TNF- α exposure in regulating the IL-6 and IL-8 level.

2 MATERIALS AND METHODS

2.1 Research Design and Ethical Approval

This study conducted at the Stem Cell and Cancer Research (SCCR) Laboratory from October to December 2019. This study used two groups: vehicle control (Veh) and a treatment (T) group. The institutional review board of the Committee ethic of Medical Faculty, Sultan Agung Islamic University of Semarang, Indonesia, approved this study.

2.2 Isolation and Culture of Human Umbilical Cord-MSCs

MSCs were isolated from umbilical cords and cord blood obtained from donors with written informed consent. The isolation and expansion of MSCs were performed, as described previously (1). Briefly, cords were cut into smaller pieces and transferred into a T25 culture flask (Corning, Tewksbury, MA, USA) containing DMEM (Sigma-Aldrich, Louis St, MO) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL)/streptomycin (100 μ g/mL) (Gibco™ Invitrogen, NY, USA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was renewed every 3 days and passaged after reaching 80% confluences (14 days). UC-MSCs-like at passages 4–6 was employed.

2.3 MSCs Characteristic

The of UC-MSCs-like cells was confirmed by analyzing MSCs specific markers and the capability to differentiate into mature cells. The 5th passage of RUC-MSCs-like was stained with fluorescence-labelled specific MSCs antibody including PE-CD44 (Clone G44-26, 555479; BD Biosciences), APC-CD73 (Clone AD2, 560847; BD Biosciences), FITC-CD90 (Clone 5E10, 561969 BD Biosciences), PerCP-CD105 (Clone 266, 560819, BD Biosciences), and PE-Lin negative (CD45/CD34/CD11b/CD19/HLA-DR) antibodies, then incubated for 30 minutes at room temperature, washed twice with stain buffer (554657, BD Biosciences) and examined using a BD C6 Plus flow cytometer (BD Biosciences) and BD Accuri C6 Plus Software (BD Biosciences).

2.4 Differentiation of hUC-MSCs

To characterize the isolated cells, we further performed the osteogenic differentiation assay in the fourth passage. Osteogenesis was induced by osteogenic induction medium containing 10 mmol/L β glycerophosphate, 10^{-7} mol/L $0.1 \mu\text{M}$ dexamethasone, $50 \mu\text{mol/L}$ ascorbate-2-phosphate (Sigma-Aldrich, Louis St, MO) and supported with 10% FBS (Gibco™ Invitrogen, NY, USA) in DMEM (Sigma-Aldrich, Louis St, MO) at 37°C and 5% CO_2 . Calcium deposition was shown by Alizarin Red staining (Sigma-Aldrich, Louis St, MO) after 21 days incubation.

2.5 Bacteria Preparation

We used *S. aureus* as the source of infection in this study. The bacteria were obtained from the Laboratory of Microbiology, Faculty of Medicine, Unissula. *S. aureus* was propagated in LB medium (BD Falcon) overnight at 37°C and used to coat mesh implanted material during the log phase of growth.

2.6 Isolation of Human Peripheral Blood Mononuclear Cells (hPBMCs)

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll-Paque (Axis-Shield) density gradient centrifugation from health volunteers' venous blood after informed consent. PBMCs were cultured in 2 ml of advanced Roswell Park Memorial Institute medium (RPMI) 1640 culture medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS, 2 mM glutamine, 100

U/ml penicillin, and streptomycin, and allowed to adhere at 37°C and 5% carbon dioxide incubator for 12 h.

2.7 Co-culturing MSCs-adhered Bacteria with PBMCs under TNF- α Stimulation

For the T group, *S. aureus* and hUC-MSCs (4×10^4 cells) were co-cultured in coverslip and put in a T25 culture flask in DMEM (Sigma-Aldrich, Louis St, MO) at 37°C and 5% CO_2 for 12 h. The co-cultured cells were then transferred to the culture flask, which contains 8×10^5 PBMCs (1:10 comparison), combined with DMEM-LG and RPMI 1640 culture medium (Invitrogen, Grand Island, NY, USA), 10% FBS, 2 mM glutamine, and 100 U/ml penicillin and streptomycin. The co-cultured cells were also supplemented with TNF- α recombinant (10 ng/mL) (BioLegend, San Diego, CA). The medium supernatant was collected after 1, 4, 8, and 12 h incubation for ELISA analysis. On the other hand, the co-culture between PBMCs and *S. aureus* was performed for the Veh group.

2.8 Quantification of Cytokines

The levels of both IL-6 and IL-8 were quantified in the cell culture supernatants by enzyme-linked immunosorbent assay (ELISA) from the various treatment groups. IL-6 and IL-8 were calculated according to a standard curve constructed for each assay, and each assay performed in triplicate. The colorimetric absorbance was recorded at a wavelength of 450 nm. The measurement was done entirely according to the manufacturer's protocol (QAYEE, Wuhan, China).

2.9 Data Analysis

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 the paired t-test. p values: *, $p < 0.05$.

3 RESULTS

3.1 Isolation and Differentiation of hUC-MSCs

Isolation of hUC-MSCs was performed based on the capacity to plastic attachment under standard culture

conditions. Isolated cells were cultured for 2–3 weeks in monolayer and used for differentiation analysis after 4 to 5 passages. The hUC-MSCs were initially characterized by their elongated fibroblastic cellular phenotype (figure 1(a)); moreover, osteogenesis was confirmed at day 21 of culture by immunodetection with Alizarin Red staining (figure 1(b)).

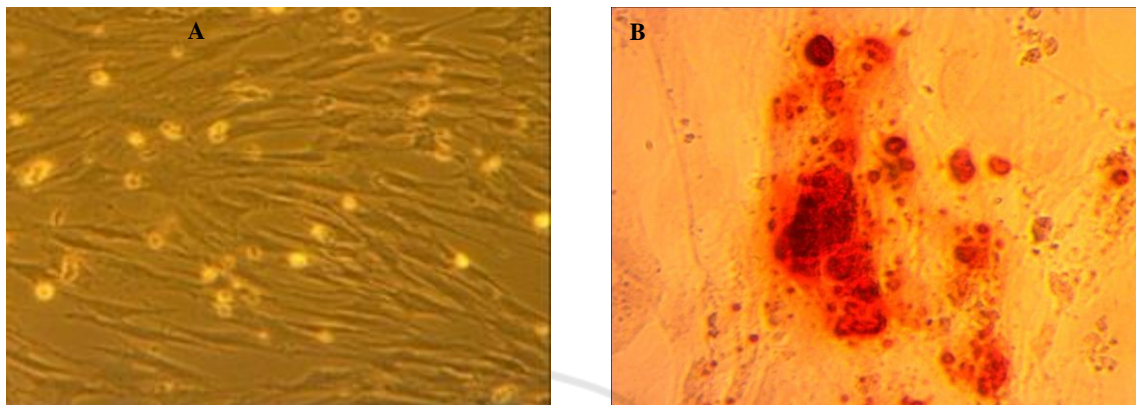


Figure 1. (a) UC-MSCs characterized by their peculiar fibroblast-like (spindle shape) morphology (100x magnification). (b) and osteogenic differentiation with Alizarin Red staining appears red color (400x magnification).

3.2 Characteristics of hUC-MSCs

According to the International Society of Cellular Therapy (ISCT), MSCs have specific marker profiles, such as CD73, CD105, CD90 and negative of Lin- (CD45/CD34/CD11b/CD19/HLA-DR) and the

capability to differentiate into several mesodermal germ layers, including osteocytes. The result showed that UC-MSCs expressed a high level of CD90 (99.4%), CD105 (96.9%), CD73 (99.8%) and lacked the expression of Lin- (1.3%). (Figure 2).

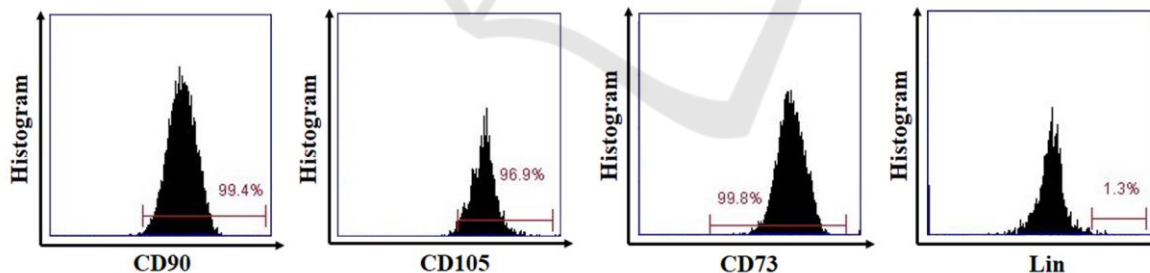


Figure 2. MSCs phenotypes was positive for CD73, CD90 and CD105 and negative for Lin.

3.3 The Appearance of MSC-Adhered Bacteria and PBMCs

We firstly co-cultured the MSCs with *S. aureus* (figure 3(a)) in 12 hours incubation to determine the IL-6 and IL-8 concentration at first-hour incubation, with and without PBMCs. Next, we co-cultured the MSCs-adhered bacteria with PBMCs and observed its appearance at 12 hours incubation (figure 3(b)).

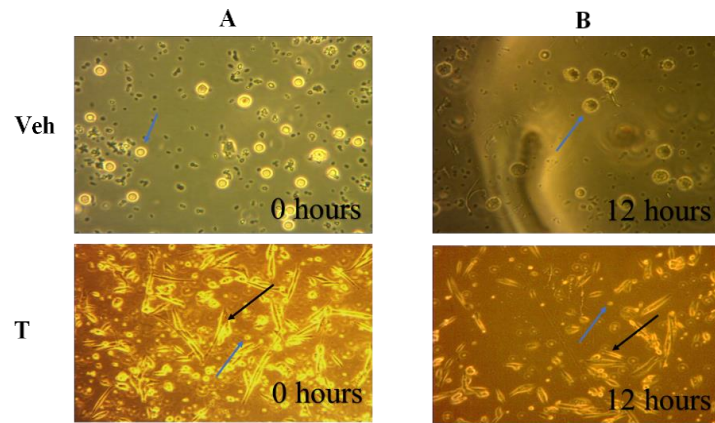


Figure 3. (a) MSCs adhered bacteria (black arrow) and PBMCs (blue arrow) appearance at 0 and (b) 12 hours incubation. (Magnification: Veh = 40x; T = 10x).

3.4 IL-6 and IL-8 Levels

We subsequently quantify the level of IL-6 and IL-8 (Figure 3) by ELISA in triplicate. The IL-6 and IL-8 level analysis showed a significant increase in the T group at the first-hour incubation ($p < 0.05$).

Interestingly, the level of IL-6 gradually decreased, depending on time, which became significant after 12-hour incubation ($p < 0.05$). In line with this, the level of IL-8 was also gradually decreased in time, which reached significant after 8- and 12-hours incubation ($p < 0.05$).

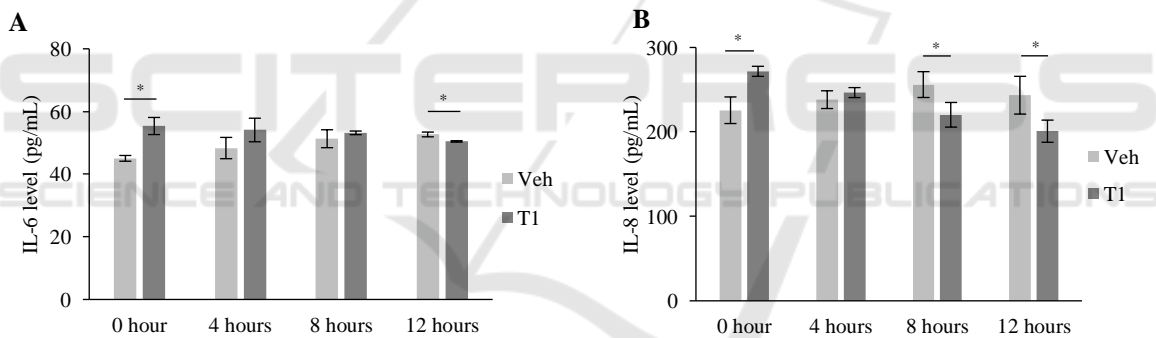


Figure 4. ELISA assays for the T group in 0,4,8 and 12 hours post-co-culture showed the gradual decrease of IL-6 and IL-8 depend on time. (a) The level of IL-6 was significantly after 12 hours of co-culture incubation. (b) In line with this, the level of IL-8 was also significantly decreased after 8- and 12-hours incubation. *, $p < 0.05$.

4 DISCUSSION

Inflammation serves as a systemic or localized protective response caused by injury, infection, or tissue destruction and attends to eliminate pathogens and preserve host integrity, particularly in sepsis model infection. MSCs may exhibit antimicrobial properties and regulate both the innate and the adaptive immune responses, resulting in both the pro-inflammatory and anti-inflammatory effects when those MSCs interact with an immune system or exposed by various cytokines (19). Several studies

have shown that MSCs respond to the inflammatory milieu by polarizing either into MSCs type-1 with the pro-inflammatory phenotype or MSCs type-2 with anti-inflammatory properties depending on Toll-like Receptors (TLRs) type activation (1,20). Another study also reported that MSCs under bacterial exposure might increase phagocytosis of neutrophil and monocyte cells, suggesting that MSCs may increase pro-inflammatory molecules (21). Although MSCs have been widely demonstrated experimentally on their immune properties in suppressing inflammation, the study of MSCs in

treating severe infections by exploring the IL-8 and IL-6 is comparatively less performed (18). Therefore, in the present study, we demonstrate the utility of activated MSC in treating the *S. aureus*-contaminated PBMC under TNF stimulation by examining the IL-8 and IL-6 levels as the one marker of severe infections.

This study found a significant increase of IL-8 level in treatment groups starting at first to 4 hours incubation on *S. aureus*-contaminated PBMC with TNF stimulation. Our finding suggested that MSCs exposed by bacteria could enhance neutrophils to recognise and kill bacteria *S. aureus* due to expressed IL-8 following MSCs treatment is the robust chemoattractant for neutrophils. This study confirmed and extended our understanding of the direct antibacterial activity of MSC as previously reported when neutrophils incubated with the activated MSCs can induce significantly greater Neutrophil Extracellular Trap (NET) area formation (22). The increased IL-8 in the first hours after exposure indicated MSCs could stimulate neutrophils, the innate immune system, to phagocytize bacteria preventing the bacteria from spreading into tissues (23). Interestingly, we also found that the IL-8 level gradually decreased in time and reached significant after 8- and 12-hours incubation. We suggest the polarization of MSCs caused the gradual decrease of IL-8 into MSCs type-2 following exposed bacteria as described in our previous study (1).

In line with the increase of IL-8, there was also a significant increase of IL-6 in treatment groups starting at first to 4 hours of incubation on *S. aureus*-contaminated PBMC with TNF α stimulation. Likewise, with 8 to 12 hours of incubation, we also found the decreased IL-6 decreased and reached significant after 12 hours incubation, similar to an episode of IL-8. We supposed that there were the indirect mechanisms of MSC in regulating IL-8 and IL-6, in which MSCs initially increase those cytokines, subsequently became decreased gradually in time. This is due to the activation of MSCs with TLRs ligand, known as MSCs polarization. In terms of bacteria stimulation and TNF exposure, the TLR-4 of MSCs type-1 was activated initially to induce the activation of MyD88-dependent pathway, then to NF- κ b pathway resulting in the release of pro-inflammatory cytokines including IL-8 and IL-6. These released cytokines trigger the inflammation states crucial for innate cells to eliminate the bacteria and antigen (24). However, along with time, the increased IL-8 and IL-6 induce the upregulation of COX2, which increases PGE2 secretion. Binding PGE2 to EP2 and EP4 receptor leads the shift of

MSCs from MyD88-dependent pro-inflammatory to TRIF-TRAM mediated anti-inflammatory signal, known as MSCs type-2 by P110 δ isoform of PI3K kinase. This MSCs type-2 can secrete anti-inflammatory cytokines such as IL-10 (1). This condition leads to decreased IL-8 and IL-6 levels.

Our findings indicate that the regulation of IL-6 and IL-8 by MSCs type-1 is needed for the neutrophils to migrate and attach to the inflammatory niche. However, the excessive release of IL-6 and IL-8 is also needed to suppress MSCs type-2 to reach the homeostasis process. Unfortunately, we did not measure the concentration of NET and a direct bacterial killing assay of MSCs and the anti-inflammatory cytokines. Therefore, the exact mechanism of MSCs polarization regarding pro-inflammatory cytokines and anti-inflammatory cytokine remains unclear.

5 CONCLUSION

Based on our study, we conclude that MSCs may regulate the IL-6 and IL-8 production on bacteria-contaminated PBMC with inflammation in early incubation to late incubation.

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