ORIGINAL PAPER

The Effect of Secreted IL-10 from Mesenchymal Stem Cell on Immune Checkpoint Molecules

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ABSTRACT

Background: Immunosuppression in sepsis is hypothesized to result from the increased expression of the immune checkpoint molecules programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1). PD-1 and PD-L1 blockade therapies have been reported to increase survival in septic animals. Currently, the interleukin (IL)-10 within mesenchymal stem cell (MSC) secretome is known for its immunomodulatory capacity. **Objective:** To study the effect of IL-10 within MSC secretome on the expression of immune checkpoints in the rat model of sepsis. **Methods:** We used 48 male Rattus norvegicus rats in this research and divided them into four groups: sham (rats without sepsis induction and treatment), control (sepsis-induced rats without treatment), T1 (sepsis-induced rats treated with 150 µL of secreted IL-10 from MSC), and T2 (sepsis-induced rats treated with 300 µL of secreted IL-10 from MSC). Forty-eight hours after sepsis induction, we terminated the rats and collected the blood to examine the PD-1 and PD-L1 expression levels. **Results:** We found a decrease in the relative expression of PD-1 in the septic rat group given 150 µL and 300 µL of secreted IL-10 from MSC compared to the control group, but the decrease was not significant. We also found a decrease in the relative expression of PD-L1 mRNA in the septic rat group given 150 µL and 300 µL of secreted IL-10 from MSC compared to the control group. **Conclusion:** Administering secreted IL-10 from MSC reduces the expression of PD-1 and PD-L1 in sepsis. These findings suggest that MSC secretome can improve the immunosuppression in sepsis.

Keywords: IL-10, immune checkpoints, immunosuppression, secretome, sepsis.

1. BACKGROUND

Sepsis is defined as a life-threatening organ failure resulting from inappropriate immune responses to infection (1). Research shows that despite a predominance of the hyperinflammatory phase in the initial phase, sepsis rapidly progresses to a state of immunosuppression. Immunosuppression is increasingly recognized as a significant contributing factor to the mortality in sepsis. One of the major mechanisms is hypothesized to be an increase in the expression of immune checkpoint molecules programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) (2,3).

In septic shock patients, PD-1 is upregulated in T lymphocytes, whereas PD-L1 is upregulated in monocytes. The expression level of PD-L1 in monocytes also correlates with the 28-day mortality rate in septic patients (4, 5). PD-1 and PD-L1 blockade therapies have been widely studied and reported to increase survival in septic animals (6, 7), indicating that the regulation of immunosuppression is crucial in sepsis management.

Currently, mesenchymal stem cell (MSC) therapy is one of the novel approaches for managing immune response dysregulation. However, this therapeutic capability originates from the bioactive factors within the MSCs: the secretome. Secretome includes two different components: the exosomes and the soluble proteins with paracrine effects involved in cell communication (8, 9). IL-10, one of the bioactive factors, plays a major role in the immunomodulatory potential of secretome (10).

2. OBJECTIVE

We aim to study the effect of IL-10 within the secretome from MSC on the expression of immune checkpoint molecules in the rat model of sepsis.

3. MATERIAL AND METHODS

Study type and ethical approval

This study is a true experimental laboratory research with post-test only control group design. Sampling was conducted using the principle of non-probability sampling in a consecutive sampling manner using Rattus norvegicus rats as the research subjects. This study was approved by the Health Research Ethics Committee with letter number 541/2022.

MSC isolation and secretome preparation

The isolation process was conducted in Universitas Islam Sultan Agung (Unissula) Faculty of Medicine Stem Cell and Cancer Research (SCCR) Laboratory. Rats with a gestational age of 21 days were anesthetized using intramuscular injection of lethal dose anesthetics, with 10 mL cocktail using 50 mg/kg body weight of ketamine, 10 mg/kg body weight of xylazine, and 2 mg/kg body weight of acepromazine. Then the umbilical cord was collected, cultured, and isolated following a standardized method in SCCR laboratory.

We performed a validation test on the MSC surface markers using flow cytometry according to the manufacturer's instructions. To stain the surface antigen, we incubated the cells with rat anti CD90.1-PerCP/CD29-APC for the MSC markers and rat anti CD31-PE/CD45-FITC for the hematopoietic markers (BD Bioscience, San Jose, CA, USA). After the staining, cells were examined and analyzed with BD Accuri C6 Plus flow cytometer and BD Accuri C6 Plus software (BD Bioscience).

We also examined the adipogenic and osteogenic differentiation potential of the MSCs. At 95% confluence, MSCs were cultured in a standard medium at 37°C and 5% CO2. Then the standard medium was replaced with a differentiation basal medium (Rat MesenCultTM, Stem Cell Technologies, Singapore) supplemented with 1% L-glutamine, 1% penicillin, and 0.25% amphotericin B (Gibco). The medium was renewed every three days. After an incubation period of 21 days, the MSCs were stained with oil red O and alizarin red staining for lipid and calcium deposits, respectively (Sigma-Aldrich, Louis St, MO).

MSCs at 80% confluence were placed in a hypoxic chamber (Stem Cell Technologies) with 5% oxygen. Oxygen partial pressure (pO2) was measured with an oxygen controller (BioSpherix, Lacona, NY, USA). The cells were incubated for 24 hours at 37°C and then collected and centrifuged at 13000 g at 4°C for 10 minutes to separate the conditioned medium. We used the Tangential Flow Filtration (TFF) method (Formulatrix, MA, USA) to isolate the secretome. The secretome was then stored at -80°C. Finally, we performed an enzyme-linked immunosorbent assay (ELISA) based on the manufacturer's instructions (Invitrogen, CA, USA) to analyze the secretome content.

Research sample

The samples were divided into four groups: sham (rats without sepsis induction and treatment), control (sepsis-induced rats + intraperitoneal imipenem/cilastatin at 25 mg/kg body weight), T1 (sepsis-induced rats + intraperitoneal

imipenem/cilastatin at 25 mg/kg body weight + 150 µL of secreted IL-10 from MSC injected intravenously into the tail), and T2 (sepsis-induced rats + intraperitoneal imipenem/cilastatin at 25 mg/kg body weight + 300 µL of secreted IL-10 from MSC injected intravenously into the tail). With the resource equation method (11-13), we obtained 4 and 6 as the minimum and maximum number of animals per group, respectively. In this study, we used 5 rats per group with an expectation of 60% attrition (11), so the final sample size was 12 rats per group. Thus the total rats in all test groups were 48.

The research sample was randomly selected using inclusion and exclusion criteria. The inclusion criteria for the rats in this study were male, aged 10-12 weeks, body weight of 200-300 g, and had not been used for other studies. We excluded sick or infected rats during the adaptation period and rats that died before the sepsis induction. The drop-out criteria of the rats in this study were rats that died immediately after the sepsis induction.

Rat rearing and sepsis induction

Rats were treated according to ethical principles: free from hunger, thirst, discomfort, pain, fear, and stress. Each rat was kept in a separate cage to obtain management conditions similar to incentive rooms. Rats were kept at room temperature (37°C) with 12/12 h light/dark cycles and given food and drink ad libitum.

Rats were adapted for 7 days. On the 8th day, we performed sepsis induction using the fecal intraperitoneal-injection (FIP) method in the control, T1, and T2 rat groups based on the method in past studies (14, 15).

PD-1 and PD-L1 expression levels examination

Forty-eight hours after sepsis induction, the rats were terminated using intramuscular administration of 80 mg/kg body weight of ketamine and 4 mg/kg body weight of xylazine. Then we collected the blood from the rats to examine PD-1 and PD-L1 expression levels.

We first converted RNA to cDNA. Then we made cocktails from PD-1 and PD-L1 solutions with these respective compositions: 10 µL of SYBR green RT-PCR mastermix, 2 µL of primer (F), 2 µL of primer (R), 2 µL of control primer (F), 2 µL of control primer (R), 5 µL of ddH2O, and 1 µL of cDNA. The relative quantitation of PD-1 and PD-L1 expression levels was analyzed using the delta cycle threshold (CT) method. The primer sequence of each gene and the RT-PCR method are based on past studies (16-18).

Statistical analysis

We perform statistical analyses on the data of PD-1 and PD-L1 with Kruskal-Wallis analysis. Then we analyze the significant data with Mann-Whitney post hoc test. $p < 0.05$ indicates significance.

4. RESULTS

MSC validation and characterization

Isolated MSC with 80% confluency under microscopic observation showed spindle-like cell morphology. Flow cytometry results showed a high expression of CD90.1 and CD29 (99.4% and 96.9%) and a low expression of CD45 and CD31 (1.3% and 6.6%). Upon staining with oil red O and alizarin red, the MSC culture showed adipogenic and osteogenic differentiation represented by the presence of lipid and calcium deposition, respectively. Using the TFF method, we obtained
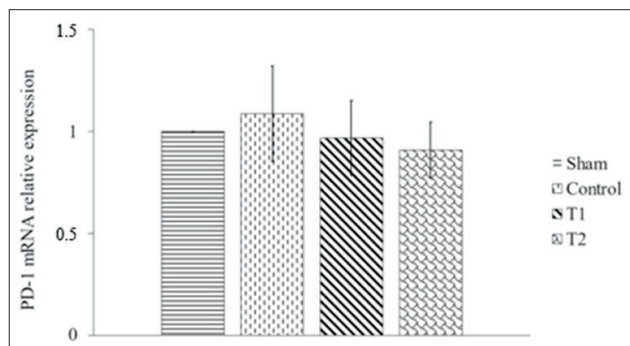
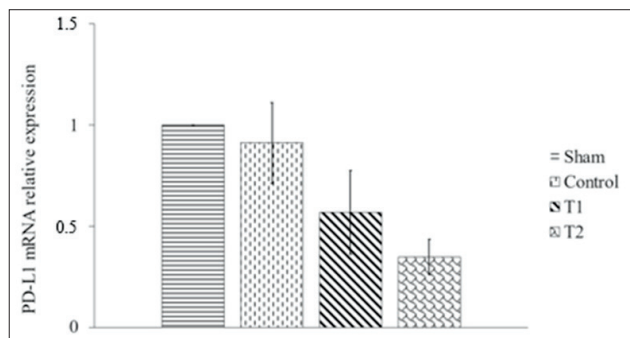**Figure 1. PD-1 mRNA relative expression levels of all rat groups**

Figure 2. PD-L1 mRNA relative expression levels of all rat groups

secretome containing 269.57 ± 38.39 pg/mL of IL-10.

Expression levels of immune checkpoint

The result of Kruskal-Wallis analysis showed no significant difference in the decrease of the relative expression level of PD-1 in the septic rat group given secretome, with $p > 0.05$ ($p = 0.379$).

Meanwhile, we found a significant difference in the decrease of the PD-L1 relative expression levels in the septic rat group given the secretome, with $p < 0.05$ ($p = 0.002$). We continued the analysis with the Mann-Whitney post hoc test. The rvhesults showed no significant difference in the decrease of the PD-L1 relative expression levels in the T1 and T2 groups compared to the control group ($p = 0.637$ and $p = 0.079$, respectively) (Figure 2).

5. DISCUSSION

The major mechanism of immune checkpoint in sepsis occurs through PD-1/PD-L1 ligation. PD-L1 is one of two ligands for PD-1 and expressed by T and B lymphocytes, dendritic cells (DCs), macrophages, and various non-hematopoietic tissue cells. PD-L1 gene deficiency is reported to improve survival in sepsis (19, 20).

Functionally, PD-1 and PD-L1 inhibit innate immune cell functions such as phagocytosis, pathogen clearance, and cytokine release. However, in severe infections, sustained PD-1/PD-L1 ligation leads to T cell exhaustion. This phenomenon reduces effector T cell function, proliferation capacity and cytokine production, and causes apoptosis (3). This results in secondary infection and death due to the failure to set up an effective immune response (21).

In this study, we found a decrease in the relative expression of PD-1 mRNA in the septic rat group given 150 μL and 300 μL of secreted IL-10 from MSC compared to the septic rat group without secretome treatment, but the decrease was not significant. We also found a decrease in the relative ex-

pression of PD-L1 mRNA in the septic rat group given 150 μL and 300 μL of secreted IL-10 from MSC compared to the septic rat group without secretome treatment. Inflammatory environment has been known to induce the expression of immune checkpoint molecules in T cells. Priming of MSCs with proinflammatory cytokines, such as TNF-α and IL-1β, has been shown to upregulate PD-1 ligands expression (22). However, MSCs are also known to possess immunomodulatory properties (23). The IL-10 from MSCs suppresses the function of macrophages and DCs, thereby reducing the production of proinflammatory cytokines (24). This may explain the decrease in the expression of immune checkpoint molecules in our study.

Interestingly, in the septic rat group given 300 μL of secreted IL-10 from MSC, the relative expression of PD-1 decreased to a lower level compared to the septic rat group given 150 μL of secreted IL-10 from MSC. Similar findings were also observed in the relative expression of PD-L1 in the septic rat group given 300 μL of secreted IL-10 from MSC. However, the relative expression of PD-L1 showed a marked decrease compared to the other sepsis-induced rat groups, perhaps due to the stronger suppression of the inflammatory environment by the higher amount of IL-10.

Although PD-1 and PD-L1 blockade therapies have been widely studied and reported to increase survival in septic animals (6, 7), excessive PD-1 and PD-L1 blockade can cause the immune system to attack healthy cells. Extremely low expression of PD-1 and PD-L1 is associated with impaired immune tolerance and immune system-mediated tissue damage (20,25). Therefore, the dose of secretome needs to be considered to avoid these side effects.

6. CONCLUSION

Administering secreted IL-10 from MSC reduces the expression of PD-1 and PD-L1 in sepsis. These findings suggest that MSC secretome can improve the immunosuppression in sepsis. However, a lower dose of MSC secretome is recommended to avoid the side effects from excessive suppression of the immune checkpoint molecules.

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