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Original Article

Mesenchymal stem cells promote infiltration of myeloid-derived suppressor cells after acute myocardial infarction via up-regulation of multiple cytokines

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Abstract: Mesenchymal stem cell (MSC) therapy improves cardiac function after myocardial infarction (MI) in part via immune modulation. Myeloid-derived suppressor cells (MDSCs), defined as cells expressing the myeloid lineage markers cluster of differentiation CD11b and granulocyte-differentiation antigen Gr-1, are presumed to be recruited to sites of inflammation. However, it is not known whether the beneficial effects of MSC therapy for MI treatment are mediated by infiltrated MDSCs in infarcted hearts. Bone marrow-derived MSCs were injected into the peri-ischemic region of the myocardium after ligation of the proximal left coronary artery in a mouse model of MI. On days 1, 3, and 7, MDSC populations in blood and infarcted myocardium were examined by flow cytometry. At 1 day after MI, the number of $CD11b^+$ $Gr\text{-}1^+$ MDSCs was increased in the ischemic myocardium of MSC-injected as compared to saline-injected mice ($P < 0.001$), whereas no differences were observed between the two groups on days 3 and 7. Histological analysis confirmed that MDSC recruitment to the myocardium was higher in the MSC-injected group than in the control group 1 day after MI. MSC treatment increased mRNA levels of chemokine (C-C motif) ligand 2 (CCL2), C-X-C motif chemokine (CXCL5) macrophage colony-stimulating factor (M-CSF), and cyclooxygenase 2 (COX2) in infarcted hearts. These results indicate that MSC treatment increases MDSC infiltration into the infarcted heart in the early period after MI via modulation of CCL2, CXCL5, M-CSF, and COX2 expression.

Keywords: Myeloid-derived suppressor cells, mesenchymal stem cell, myocardial infarction

Introduction

Acute myocardial infarction (MI) is a major complication of coronary heart disease and is associated with a high mortality rate [1]. Structural and functional remodeling of the heart after MI involves an inflammatory response, followed by scar formation at the site of infarction as well as changes in the remote myocardium such as fibrosis and vascular remodeling. Mesenchymal stem cell (MSC) transplantation is among the most promising therapies for MI [2], as these cells can be induced to differentiate into cardiomyocytes and vascular cells [3]. Moreover, MSCs may modulate the immune response and thereby promote tissue repair. Recent reports suggest that MSCs mediate these therapeutic effects in a paracrine manner [4-6] by suppressing the immune response via inhibition of dendritic cell maturation [7] and T and B lymphocyte and natural killer (NK) cell functions [8] in autoimmune and inflammatory diseases [9].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells

that accumulate under various pathological conditions. MDSCs have an immunosuppressive function and are characterized by co-expression of cluster of differentiation CD11b and granulocyte-differentiation antigen Gr-1 in mice [10]. They also regulate the innate immune response controlled by NK cells [11] and macrophages. Furthermore, they suppress adaptive immunity by inhibiting T cell function [12], which is mechanistically linked to arginase 1 [13] and inducible nitric oxide synthase expression and reactive oxygen species production [10] by MDSCs. These cells have been detected in cancer patients [14] and tumor-bearing mice [15], and have also been found to be enriched in acute inflammation [16], experimental autoimmune uveitis (EAU) [17], liver injury [18], graft-versus-host-disease models [19], myocarditis [20], and hypertension [21]. However, the precise role of MDSCs in MI has not yet been determined.

We speculated that MSC infusion following MI induces MDSC recruitment in the infarct region and decreases inflammation, thereby reducing post-MI ventricular remodeling. To test our hypothesis, we evaluated the presence of MDSCs in the organs of mice treated with MSCs after MI. We also assessed the expression of cytokines involved in the recruitment and expansion of MDSCs. The findings provide insight into the mechanism of MSC-mediated immune regulation in MI, as well as a basis for improved design of MSC-based therapies.

Materials and methods

Mouse bone marrow-MSC isolation

Bone marrow was isolated from male C57BL/6 mice (7 weeks old) that were purchased from KOATEC (Korea). The femur and tibia of the mice were excised and all connective tissue attached to the bones was removed. Bone marrow plugs were extracted from the bones by flushing the bone marrow cavity with Dulbecco's Modified Eagle Medium. After a homogenous cell suspension was achieved, the cells were centrifuged and resuspended in complete culture medium. MSCs were cultured in low-glucose Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% antibiotics-antimycotics (Gibco, USA). After 1 day, non-adherent cells were removed and fresh medium was added to the cultures. The medium was replaced every 2 days and cells were sub-cultured at a 1:10 ratio. Cells from passage 7-9 were used for experiments.

Mouse MI model

Animal study protocols were approved by the Institutional Animal Care and Use Committee of Catholic University (CUMC-2016-0008-01). Mice were maintained under specific pathogen-free conditions in an animal facility with controlled light, temperature, and humidity. The air in the facility was passed through a high-efficiency particulate-arresting filter system designed to exclude bacteria and viruses. Male C57BL/6 mice (7 weeks old, 20-22 g) were anesthetized by intraperitoneal injection of Zoletil and Rompun in saline (0.2 ml/100 g). Anesthesia was maintained via mechanical ventilation (Harvard Apparatus, USA) with supplemental oxygen. Mice were intubated with an Angiocath Plus 22GA catheter (BD Biosciences, USA) and placed on an operating table. The muscles were removed and the ribs were fixed using 5-0 nylon silk and the left anterior descending coronary artery (LAD) was ligated using 8.0 silk. Before chest closure, the air was removed using the Angiocath plus. Body temperature was maintained with an infrared lamp. Mice were sacrificed 1, 3, 7, and 28 days after MI for analysis.

Echocardiography

Echocardiography was performed using an Affinity 50 imaging system (Philips) 28 days after MI. Mice were anesthetized with 5% isoflurane initially and then with 1% isoflurane during the echocardiography procedure to maintain the heart rate. Ejection fraction (EF) and fractional shortening (FS) were calculated from M-mode tracings at the level of papillary muscles to enable consistent measurement at the same anatomic location in different mice.

Flow cytometry analysis

A single cardiac cell suspension was prepared using a MACS C tube (Miltenyi Biotec, USA), as previously described [22]. Red blood cells (RBCs) were isolated with RBC lysis buffer (Qiagen, USA). Heart tissue was digested with collagenase type II solution (Worthington Biochem, USA) at a concentration of 500 U/ml in 37°C for 40 min; cells were then passed through a 40-μm cell strainer and Hank's Ba-

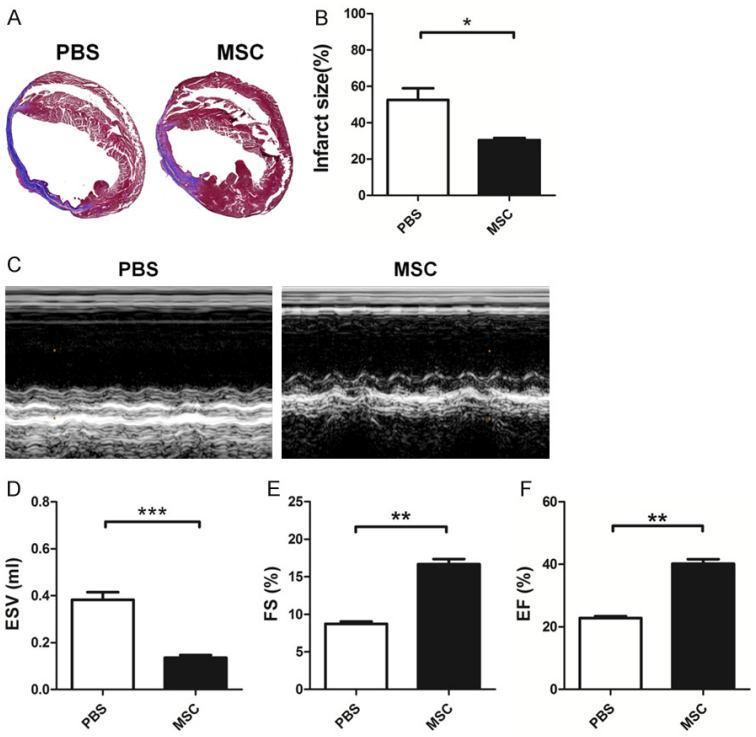

Figure 1. MSC injection in a mouse model of MI reduces infarct size after 28 days. A. Masson's trichrome staining of heart tissue revealing left ventricular fibrosis at 28 days after MI. Mice were injected with PBS (control) or MSCs. Cells (2×10^5 diluted in 20 µl PBS) were directly injected into the peri-infarct area after MI. Red and blue indicate viable myocardium and fibrosis caused by infarction, respectively. B. Quantitative analysis of infarct size at 28 days after MI (n = 4 per group). ***P < 0.001 vs. control group (unpaired t test). C. Representative M-mode images from mouse heart at day 28 after MI. D-F. Quantitative group echocardiography data for end systolic volume (ESV), fractional shortening (FS), and ejection fraction (EF) in the PBS and MSC groups at day 28 after MI.

lanced Salt Solution was added to terminate enzymatic digestion. Single cells were resuspended in fluorescence-activated cell sorting (FACS) staining buffer and total cell number was counted using an EVE cell counter (NanoEnTek, Korea). Cells were labeled with PE-conjugated anti-mouse/-human CD11b (Biolegend, USA), Percp-Cy 5.5-conjugated anti-mouse Gr-1 (eBioscience, USA), and FITC-conjugated anti-mouse CD45 (BD Biosciences) antibodies. Cells were sorted by flow cytometry on a FACS Canto II system (BD Biosciences) using FlowJo software (Tree Star, USA). The gating parameters for heart MDSCs have been previously described [23].

Immunocytochemistry

The heart was excised, rinsed in PBS, and embedded in FSC 22 frozen section media (Leica Surgipath, USA). Frozen tissue blocks were cut into 5-µm serial sections for immunocytochemical analysis. The sections were washed three times with 0.1% Triton X-100 in PBS for 5 min, and then fixed with methanol for 10 min. After blocking in 10% normal goat serum (Vector Laboratories, USA), sections were incubated overnight at 4°C with PE-conjugated anti-mouse/-human CD11b and Alexa 488-conjugated anti-mouse Gr-1 (Biolegend, USA) antibodies in PBS. On the second day after washing, sections were counterstained with DAPI and then mounted with DAKO Fluorescence Mounting Medium (Dako, USA). Cells were visualized using a confocal microscope (Zeiss, Germany), and images were processed using ZEN 2009 Light Edition software (Zeiss).

Histology

Fixed heart tissue embedded in paraffin was sectioned at a thickness of 5 µm using a rotary microtome. Sections were subjected to Masson-Trichrome staining to visualize nuclei, cytoplasm, and collagen. Images from randomly selected fields distributed across each section were acquired with a slide scanner (Leica Microsystems, Germany), and morphometric analysis of collagen tissue deposition was performed with ImageJ software (National Institutes of Health, USA).

Quantitative real-time (qRT) PCR

Harvested heart tissue was homogenized using an liquid nitrogen, and total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was quantified using a Nanodrop instrument (Thermo Fisher Scientific, USA). Reverse transcription was performed with 1 µg of total RNA in a to-

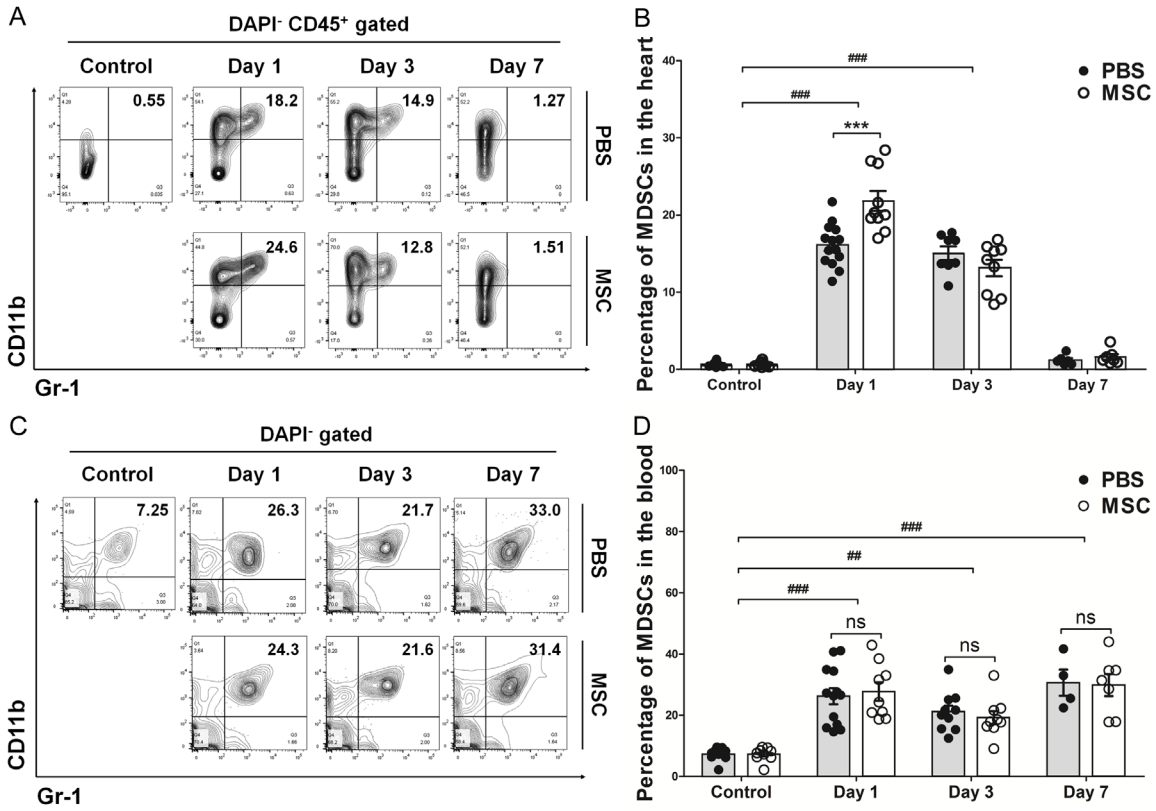

Figure 2. MDSC recruitment after MI, as assessed by flow cytometry. A. The gating strategy was used to identify MDSC populations by flow cytometry. DAPI staining was used to exclude dead and apoptotic immune cells when $CD45^+$ leukocytes were gated. MDSCs were gated as DAPI-negative cells and CD45 positive cells. MDSCs were characterized as CD11b and Gr-1 positive cells. Representative plots are shown for MDSC populations in the infarcted heart at 1, 3, and 7 days in mice injected with PBS (upper) and MSCs (lower). B. Analysis of heart tissue showing an increase in $CD11b^+$ $Gr\text{-}1^+$ cells 1 day after MI, followed by a decrease. MSC injection increased the percentage of MDSCs in the heart. C. MDSC expression in blood as evaluated by flow cytometry based on staining with DAPI and antibodies against CD11b and Gr-1. MDSC expression was increased in blood after MI and was similar in mice injected with MSCs (lower) and PBS (upper). D. Analysis of $CD11b^+$ $Gr\text{-}1^+$ cell number over time (n = 7-10 per group). ***P < 0.001 (two-way analysis of variance with the Bonferroni post-hoc test).

tal volume of 20 µl using a cDNA synthesis kit (Roche Diagnostics, Indianapolis, USA) according to the manufacturer's instructions. qRT-PCR was performed using a Light-cycler 480 system (Roche Diagnostics). Gene expression was determined as fold induction over the internal control GAPDH. Forward and reverse primer sequences were as follows: GAPDH, 5'-ATC ATC CCT GCA TCC ACT-3' and 5'-ATC CAC GAC GGA CAC ATT-3'; CCL2, 5'-CTC ACC TGC TGC TAC TCA TTC-3' and 5'-TTA CGG CTC AAC TTC ACA TTC A-3'; CXCL5, 5'-GCA TTT CTG TTG CTG TTC ACG CTG-3' and 5'-CCT CCT TCT GGT TTT TCA GTT TAG C-3'; M-CSF, 5'-AGT ATT GCC AAG GAG GTG TCA G-3' and 5'-ATC TGG CAT GAA GTC TCC ATT T-3'; and COX2 5'-TGG GCC ATG GAG TGG ACT T-3' and 5'-GGA TGT GAG GAG GGT AGA TCA TCT-3'.

Statistical analysis

Data are presented as the mean ± SEM and were analyzed using Data PRISM v.5.0 software. Mean differences were evaluated using unpaired two-tailed Student's t test or one- or two-way analysis of variance with a Bonferroni multiple comparisons post-hoc test. $P < 0.05$ was considered statistically significant. The results are representative of more than three independent experiments.

Results

MSC injection following MI ameliorates cardiac remodeling and improves cardiac function

To confirm the effect of MSCs, we compared infarct size in a mouse model of MI with or with-

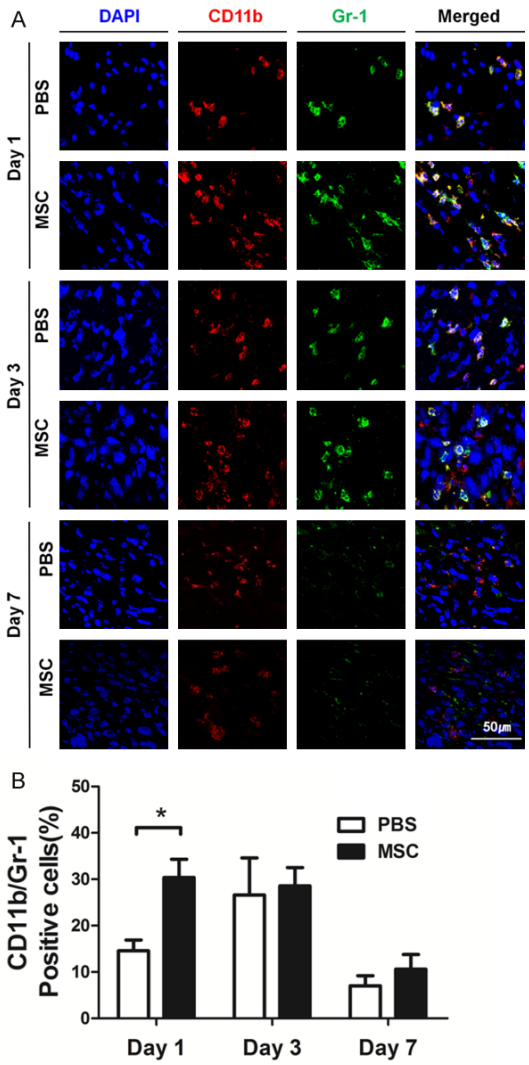

Figure 3. Infiltration of CD11b$^+$ Gr-1$^+$ cells into the infarcted heart. A. Representative images of CD11b (red) and Gr-1 (green) expression in MDSCs in the infarct border zone at 1, 3, and 7 days post-MI. Nuclei are stained with DAPI (blue). Scale bar = 50 μm (80× objective). B. Quantitative analysis of CD11b$^+$ Gr-1$^+$ double-positive cells in MSC- and PBS-injected mice (n = 4-9 per group). *P < 0.05 vs. PBS-injected group (two-way analysis of variance with a Bonferroni post-hoc test).

out intramyocardial injection of MSCs obtained from bone marrow. At 4 weeks, infarct size was reduced in mice treated with MSCs as compared to those treated with PBS (**Figure 1A**), which was confirmed by quantitative analysis (30.44% ± 1.154% vs. 52.53% ± 6.4%; P < 0.05) (**Figure 1B**). To evaluate the effects of MSCs in cardiac functions, echocardiography was performed at 28 days following transplantation. Fractional shortening and the ejection fraction were increased significantly (**Figure 1E**, **1F**), whereas end-systolic volume was decreased in the MSC group compared to the PBS group (**Figure 1D**). These results indicate that the transplantation of MSCs can ameliorate myocardial fibrosis and improve cardiac function.

MSCs stimulate MDSC infiltration into the heart in the early post-MI phase

To clarify the effects of MSCs on MDSCs after MI, we performed a time course analysis of CD45$^+$ CD11b$^+$ Gr-1$^+$ cells in the heart and CD11b$^+$ Gr-1$^+$ cells in the blood after LAD ligation with or without MSC injection. FACS analysis showed an increase in the number of MDSCs in the heart 1 and 3 days post-MI (**Figure 2A**). Moreover, the percentage of MDSCs in the heart was significantly higher in the MSC-injected as compared to the PBS-injected group on day 1 post-MI but not on later days (16.02% ± 1.114% vs. 21.80% ± 1.286%; P < 0.001) (**Figure 2B**). In contrast, the percentage of MDSCs in the blood increased gradually after MI (**Figure 2C**, **2D**), although there was no significant difference between the two groups. These results indicate that MDSCs infiltrate into the infarcted heart after MI and that MSC injection promotes MDSC recruitment in the early post-MI phase.

MDSC cells are present in the infarcted region following MSC injection

To confirm the findings described above, we evaluated CD11b and Gr-1 expression in the ischemic border zone of hearts harvested from mice 1, 3, and 7 day after MI. Transplanted MSCs are mainly incorporated into the border zone [24, 25]; moreover, the infarct border zone acts as a barrier that prevents the expansion of inflammation [26, 27]. We therefore expected that MDSCs would be recruited into this area. MDSCs were enriched in the infarct border zone after MI; the number of MDSCs was higher in mice injected with MSCs than in those injected with PBS, as determined by immunocytochemistry (**Figure 3A**). Quantitative analysis showed that the percentage of MDSCs was 2-fold higher in MSC-injected mice as compared to PBS-injected mice on day 1 (**Figure 3B**). These data are consistent with the results of flow cytometry analysis (**Figure 2A**), and indicate that transplanted MSCs induce the infiltra-

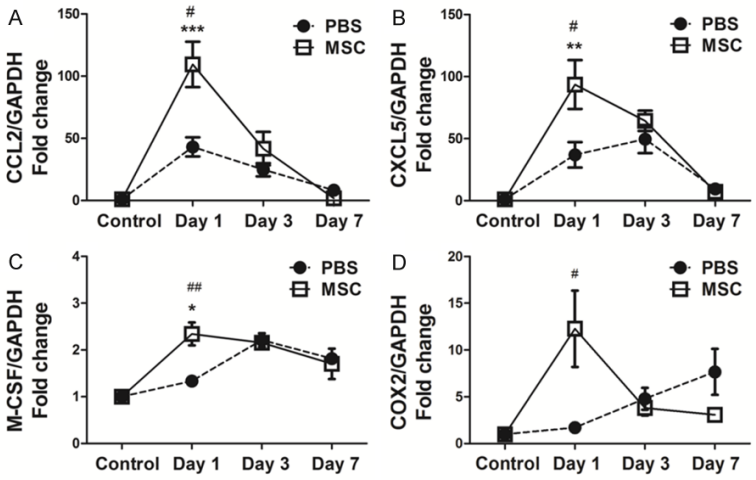

Figure 4. Upregulation of MDSC-related genes in mouse heart following MSC injection. Gene expression in the infarcted heart was analyzed 1, 3, and 7 days after MI. Target gene expression was normalized to that of GAPDH. (A) CCL2, (B) CXCL5, (C) M-CSF, and (D) COX2. Data are presented as the mean ± SEM (n = 3-7 per group) of experiments performed in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control (one-way analysis of variance); #P < 0.05 vs. PBS-injected group (unpaired t test).

tion of $CD11b^+$ $Gr\text{-}1^+$ MDSCs into the infarcted myocardium.

MSCs regulate the expression of MDSC-related genes in MI

To investigate the mechanism underlying MSC-mediated regulation of MDSCs, we examined the gene expression profile of cytokines known to recruit or stimulate the expansion of MDSCs, including *CCL2*, *CXCL5*, *M-CSF*, and *COX2*, on days 1, 3, and 7 after MI. *CCL2* expression was rapidly induced upon MI (50 fold over the control) and was 3-fold higher in MSC-injected hearts than in PBS-injected hearts on day 1 (**Figure 4A**). However, the levels on days 3 and 7 were comparable in the two groups. *CXCL5* expression was also induced in the infarcted heart, and this effect was enhanced by MSC injection at 1 day post-MI (**Figure 4B**). Similarly, the expression of *M-CSF*, and *COX2* was increased in MSC-injected as compared to PBS-injected mice on day 1 (**Figure 4C**, **4D**). These data suggest that MSCs stimulate MDSC infiltration via the release of MDSC-inducing cytokines in the infarcted heart.

Discussion

In the present study, we found that MDSCs accumulated in the heart post-MI. MSC injection induced MDSC recruitment to the infarct area via stimulation of MDSC-related genes (i.e., *CCL2*, *CXCL5*, *M-CSF*, and *COX2*), thereby reducing MI injury. These results suggest that enhanced infiltration of MDSCs could be a novel mechanism through which MSC therapy reduces myocardial ischemia injury.

MDSCs exhibit immunosuppressive activity that facilitates tumor progression by inhibiting anti-tumor immunity [15, 28]. As such, MDSC inhibition has been investigated as a promising strategy for cancer immunotherapy [29-31]. Recent studies suggest that MDSCs have beneficial effects against various inflammation-associated diseases such as inflammatory bowel disease [32], autoimmune uveitis [17], collagen-induced arthritis [33], and multiple sclerosis [34]. Under pathologic conditions, MDSCs infiltrate into tissue and suppress immune responses. These cells also play a critical role in resolving inflammation and promoting tissue repair after spinal cord injury; depletion of MDSCs was shown to impair functional recovery after injury, whereas their transplantation stimulated tissue regeneration [16].

MI triggers the inflammatory response necessary for repair of the infarcted heart [35]. However, excessive inflammation leads to cardiac remodeling. We therefore expected that MDSCs would be involved in the regulation of post-MI inflammatory responses. Indeed, a FACS analysis showed that MI induced MDSC infiltration into the infarcted heart; MDSCs started to accumulate on day 1, and their numbers remained high until day 3. These data are consistent with previous reports demonstrating an increase in $CD11b^+$ $Gr\text{-}1^+$ myeloid cells in the heart at 24 h post-MI [24, 25]. Interestingly, the number of infiltrated MDSCs decreased on day 7 post-MI, suggesting that MDSCs are involved in the early inflammatory rather than the later reparative phase. MDSCs have been shown to regulate the inflammatory response during wound healing. In a spinal

cord injury model, MDSC deficiency increased inflammation at the site of injury and consequently exacerbated tissue damage [16]. MDSCs also play a beneficial role in cardiovascular disease; in a mouse hypertension model, MDSC numbers were increased in the blood and spleen, which suppressed inflammation and reduced blood pressure [21]. In addition, Su et al. reported a role of MDSCs in viral myocarditis [36], where myocarditis resulted in enrichment of myocardial MDSCs, and adoptive transfer of MDSCs alleviated virally induced myocarditis via activation of regulatory and $CD4^+$ interleukin-10^+ T cells [36]. Additional studies are required to determine whether adoptive transfer of MDSCs can be an effective treatment for MI.

The major finding of this study was that MSCs regulate the post-MI immune response by recruiting MDSCs. MSCs play an immunomodulatory role in tissue repair and regeneration [37, 38]. Our observation that the number of MDSCs was increased by direct myocardial injection of MSCs after MI is in agreement with previous findings that intravenous infusion of MSCs recruits MDSCs in the draining lymph node, thereby ameliorating EAU in a mouse model; this effect was abolished by CCL2 knockdown [17]. In our study, we identified cytokines that may be involved in MDSC recruitment following MI. Interestingly, the expression of these cytokines was altered in the early phase but not in the late phase, corresponding to the enhanced MDSC infiltration observed only in the early phase. This result suggests that MSCs recruit MDSCs immediately after MI; however, it could also be explained by poor survival of transplanted MSCs, as cells directly injected into the ischemic region have a limited blood supply [39]. A recent report showed that a hydrogel system could improve MSC engraftment efficiency within the infarcted myocardium [40]. Thus, a hydrogel system could also increase the efficacy of MSC therapy.

Cytokines/chemokines secreted by MSCs interact with other cellular effectors to regulate cell migration, differentiation, inflammation, and proliferation [41, 42]. The expansion, activation and accumulation of MDSCs in peripheral tissues can be influenced by several factors produced by pathological conditions. We investigated the expression of MDSC-related genes in the infarcted heart. CCL2 plays a role in MDSC migration, as depletion of CCL2 signaling inhibited MDSC migration in a tumor model [43]. CCL2 has been shown to be upregulated in MI models, depletion of CCL2 reduced the infiltration of macrophages in infarcted hearts [44], and CCL2 KO mice showed delayed cardiomyocyte replacement [45]. We found that the CCL2 level was increased by MSC injection in mice with MI. Another study reported that MSC treatment combined with CCL2 silencing failed to induce MDSC recruitment and prevent EAU development, suggesting that CCL2 has an immunosuppressive effect [17]. We therefore speculate that MSC administration can release CCL2, which may be responsible for recruitment of MDSCs and promote cardiomyocyte replacement in the infarcted myocardial region. Thus, an increased number of MDSCs may contribute to the resolution of inflammation and ameliorate cardiac function post MI via CCL2.

The COX2 enzyme is involved in the conversion of arachidonic acid to PGE2, and the level of PGE2 is considered to indicate the activity of cyclooxygenase [46]. PGE2 is known as a pro-inflammatory mediator that is overproduced at sites of inflammation. PGE2 inhibits Th1 and NK cells, but enhances Th2, Th17, and Treg responses [47]. PGE2 has also been reported to control MDSC accumulation through induction of CXCL12-CXCR4 pathway [48]. PGE2 leads to induction of typical MDSC-associated suppressive factors such as IL-10, IDO1, NOS2, and IL-4Rα [49]. Moreover, secretion of PGE2 contributes to the immunomodulatory effect of MSCs [50]. PGE2-treated MSCs have been investigated with regard to promotion of cell survival and improvement of cardiac function [51]. We found that the MSC treatment group showed increased COX2 expression post MI. Administration of MSCs to the EAU mice also resulted in increased COX2 expression and number of MDSCs [17]. Our results suggest that injection of MSCs enhances COX2 expression in infarcted hearts, which may also contribute to promotion of PGE production. PGE2 generated by COX2 activity contributes to MDSC accumulation in inflamed tissue and improves damaged heart function.

In conclusion, our findings demonstrate that MDSCs are recruited to the site of injury in MI by multiple cytokines secreted by MSCs and infiltrate the infarcted area, thereby contribut-

ing to the resolution of post-MI inflammation and tissue repair. These findings provide insight into the therapeutic effects of MSCs in MI and suggest that therapeutic strategies targeting MDSCs could be effective for treatment of cardiovascular diseases.

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Disclosure of conflict of interest

None.

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