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Imaging the survival and utility of pre-differentiated allogeneic MSC in ischemic heart

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ABSTRACT

The aim of the study is to track the survival and utility of mesenchymal stem cells (MSCs) and pre-differentiated MSCs in allogeneic infarcted myocardium. MSCs labeled with green fluorescent protein and luciferase (GFP–Fluc) were characterized by flow cytometry and multi-differentiation. 5-Azacytidine (5-AZ) was employed to induced cardiac differentiation from MSCs. Cardiac markers and immune antigen expression were assessed. Then, pre-differentiated MSCs induced by 5-AZ were intramyocardially injected into allogeneic C57 mice of myocardial infarction, undifferentiated MSCs were transplanted as control. The survival of transplanted cells, immune response and cardiac function of recipients were assessed with bioluminescence imaging, immunohistochemistry and echocardiography, respectively. *In vitro* results showed that 5-AZ treatment induced cardiac differentiation from MSCs, which also increased their expression of MHC-Ia and MHC-II. After intramyocardial transplantation in allogeneic mice, 5-AZ treated MSCs would rapidly be recognized and excluded by recipients. Meanwhile, a severe infiltration of immune cells could be detected. Though beneficial effects on cardiac function by 5-AZ treated MSCs could be detected, it was short and disappeared within 1 month. In contrast, undifferentiated MSCs were immune-privileged and could survive in allogeneic myocardium for more than 1 month, resulting in a significant improvement on cardiac function.

1. Introduction

Mesenchymal stem cells (MSC) represent a promising cell source for cellular cardiomyoplasty [13,14,15]. They are widely distributed in mammals, including bone marrow, adipose tissue and umbilical cord blood, *etc* [2,6,19,27]. In the past years, many groups have investigated the potential of MSC to prevent cardiac dysfunction after myocardial infarction (MI) and confirmed that transplantation of MSCs were effective for myocardial protection [3,7,23,24].

In clinical practice, the ideal donor cells would be autologous considering the immune reaction. In term of autologous transplantation, MSCs possessed a significant advantage due to their abundant existance in mammals [6]; [26]. However, the use of autologous stem cells is usually time-consuming for isolation and expansion before transplantation [21]. Moreover, in the elderly or cancer patients, the autologous cell source may be unsuitable for therapeutic application. In such cases, an allogeneic cell source of a low immunogenicity (from young and healthy donors) would be required [8]. Allogeneic MSCs provide a good candidate, because they express a very low level of MHC class I and even no MHC class II was expressed [10,22]. In fact, different groups have studied the effects of allogeneic MSCs on cardiac function in animals after MI [5,8], but inconsistent results were acquired. Several studies reported that allogeneic MSCs were immunosuppressive *in vivo* and effectively restored cardiac function of animal models with MI [1,20], while several other studies found that these cells would be rapidly rejected by allogeneic recipients [8,17]. So, the efficacy of allogeneic MSC on ischemic hearts is still in controversy.

In terms of myocardial repair, it was considered that limited cardiac differentiation of MSCs *in vivo* was a main shortage. Therefore, several previous studies have adopted 5-azacytidine (5-AZ) treatment before transplantation to improve MSC-based myocardial repair [12,16], hypothesizing that MSC-based myocardial regeneration may be enhanced. In the study, we provided direct evidence on the survival and utility of allogeneic MSCs by using an accurate and visualized method. The immune antigen expression of MSCs was assessed *in vitro* after treatment with 5-AZ. Further, both MSCs and 5-AZ treated MSCs were intramyocardially transplanted into allogeneic recipients. The survival of transplanted cells was longitudinally tracked with bioluminescence imaging. Their immunogenicity *in vivo* and beneficial effect on cardiac function were assessed by immunohistochemistry and echocardiography, respectively.

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2. Materials and methods

2.1. Cultivation and characterization of mouse MSCs

Bone marrow mesenchymal stem cells were of BALB/C mice origin. The cells were labeled with luciferase (Luc) and green fluorescent protein (GFP). It is commercially available (Cyagen Co., Ltd., Guangzhou, China). MSCs were cultured in α-MEM containing 10% fetal bovine serum (Gibco) in 37 °C, 5% CO_2 environment.

After expansion, mouse MSCs were characterized by flow cytometry and multi-directional differentiation as previous reports [14]. For flow cytometry, anti-mouse CD90, CD105, CD45, CD34 (BD) were used. Adipogenic and osteogenic differentiation were performed to confirm the pluripotency of expanded MSCs. The verification of adipogenic and osteogenic differentiation were performed by Oil Red O staining and Alizarin Red staining, respectively.

2.2. Cardiac differentiation and immune antigen expression in vitro

To induce cardiac differentiation, MSCs were cultured in differentiation medium containing 5-AZ as previous report [8]. Differentiation was analyzed by immunochemistry and RT-PCR for the expression of cardiomyogenic markers as described below. Cardiac protein cTnT and immune antigen MHC-Ia and MHC-II were used in immunohistochemistry. For quantitative analysis of MHC-Ia and MHC-II, flow cytometry was performed.

2.3. Myocardial infarction and cell transplantation

The animals were purchased from the Experimental Animal Center, Academy of Military Medical Science (Beijing, PRC). All experiments are approved by the Institutional Animal Care and Use Committee (IACUC) of our hospital.

Myocardial infarction was induced in Male C57BL/6 mice according to the previous report [18]. Briefly, 8–12 weeks old mice were anesthetized by intraabdominally injection of sodium pentobarbital (30 mg/kg). Then, the animals were incubated and ventilated by a volume-regulated respirator in the process of surgery. Thoracotomy was performed, left coronary artery was identified and ligated with a 6-0 prolene suture. Successful ligation could be confirmed by the typical color change of left ventricular wall. 5×10^5 MSCs in 10 μL PBS were intramyocardially injected at two sites. The chest was closed and the survived mice were maintained on standard protocol.

2.4. Non-invasive imaging

For *in vitro* imaging, MSCs of different numbers, 0.1, 0.2, 0.4, 0.6, 0.8 and 1×10^6/well, were seeded onto 96 well plate in 100 μL medium (10% FBS), respectively. 300 μg/mL D-luciferin (Sciencelight) solution was prepared in α-MEM. For every 100 μL culture medium (in a 96 well plate), 100 μL D-luciferin solution (30 μg/mL) were added at room temperature. A highly sensitive charge-coupled device (CCD) camera (IVIS 50, Xenogen) was used to detect the bioluminescent signals.

For *in vivo* imaging, mice were intraperitoneal injection with D-luciferin (150 mg/kg body weight) and anesthetized with 1.5–2% isoflurane. The animals were placed in the imaging chamber and BLI signals were detected by creation of polygonal regions of interest (ROIs) over the pericardium. Images were acquired using a 2 min interval until peak signal was observed.

2.5. Echocardiography

At 4 weeks after transplantation, echocardiography was performed to assess the cardiac function as the previous described [18]. For each mouse, left ventricular ejection fraction (%, LVEF) and left ventricular fractional shorting (LVFS) were measured. Data was collected and analyzed by a blinded investigator.

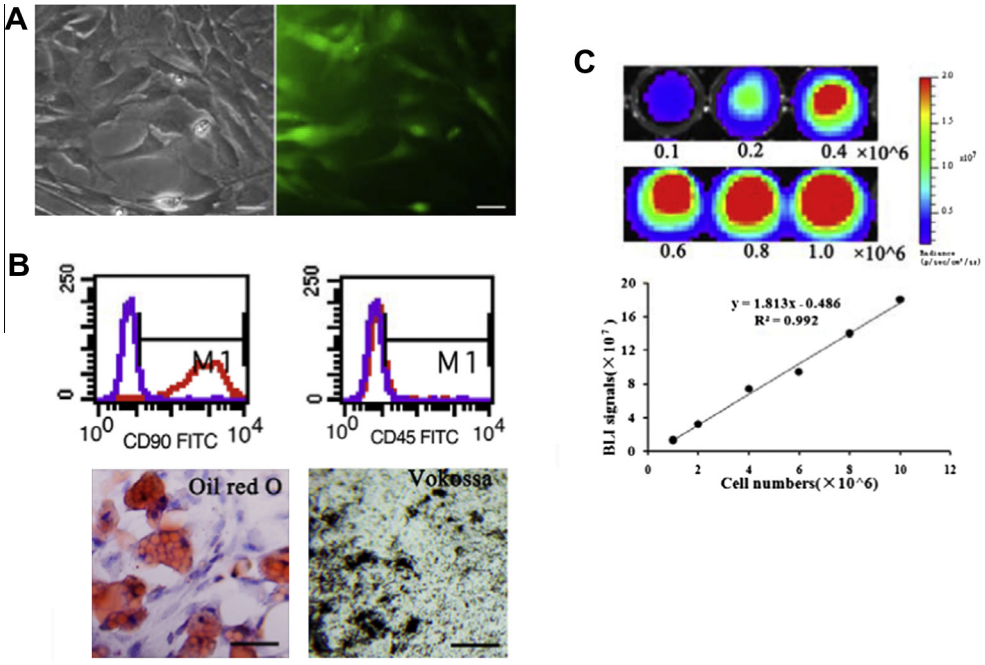

Fig. 1. Cultivation and characterization of mouse MSCs. A, spindle-shaped morphology of mouse MSC in culture and GFP expression (bar = 50 μm); B, characterization of mouse MSCs on their immune phenotype and pluripotency. The immune penotype was analyzed by flow cytometry, while the pluripotency was verified by adipogenic (Oil red O staining) and osteogenic (Vokossa staining) differentiation (bar = 50 μm); C, *In vitro* BLI reveals the strong linear relationship between cell numbers and BLI signals (R^2 = 0.992), suggesting that the reporter Fluc could be used for quantitatively tracking transplanted cells.

2.6. Immunohistochemistry

For cell samples, they were fixed in 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100, the cells were incubated with the primary antibody against cTnT (Sigma) overnight at 4 °C. After removing the redundant antibodies by washing, the cells were incubated with secondary antibodies for 2 h at room temperature. Then, the sample was stained with DAB kit.

For tissue sample, mice were sacrificed at 4 weeks, the hearts were explanted and fixed in 4% paraformaldehyde. Then, 4 μm paraffin-embodied sections were prepared according to the standard protocols. Immunostaining was performed using CD3 and CD8 antibodies, then incubated with peroxidase-conjugated streptavidin and stained with DAB. All the samples were observed under an Olympus fluorescent microscopy.

2.7. RT-PCR

Total RNA from cells was extracted with RNAprep pure Cell/Bacteria Kit (TIANGEN, Beijing, China) according to the manufacturer's instruction. cDNA was synthesized by reverse transcription using standard procedures. Cardiac genes and immune antigen genes were detected in the following PCR. The genes including Nkx2.5, β-MHC, MyoD, MHC class I, MHC class II and GAPDH. The primers were designed using primer3 online.

2.8. Statistical analysis

All the data was expressed as mean ± SD. Statistics was performed using SPSS17.0. Comparison between different groups was performed using student *t* test. Comparison between groups over time was performed by two-way repeated-measures ANOVA. $p < 0.05$ was considered significant.

3. Results

3.1. Culture and characterization MSCs

During culture, spindle shaped adherent cells could be observed and they formed symmetric colonies (Fig. 1A). After expansion, the cells were collected and immunostained for flow cytometry analysis of immune phenotype. We found that most cells were positive for CD90, CD105, and negative for CD45, CD34, *ect* (Fig. 1B). The immunophenotypes of these cells were consistent with the previous reports [9,13]. Under fluorescent microscope, green fluorescence protein could be observed in most cells (Fig. 1A). Adipoigenic and osteogenic differentiation showed that mouse MSCs possessed the capacity to differentiate into multiple lineages. Further, we assessed the activity of fluc, a reporter for bioluminescence imaging (BLI). As shown in Fig. 1C, *in vitro* bioluminescence

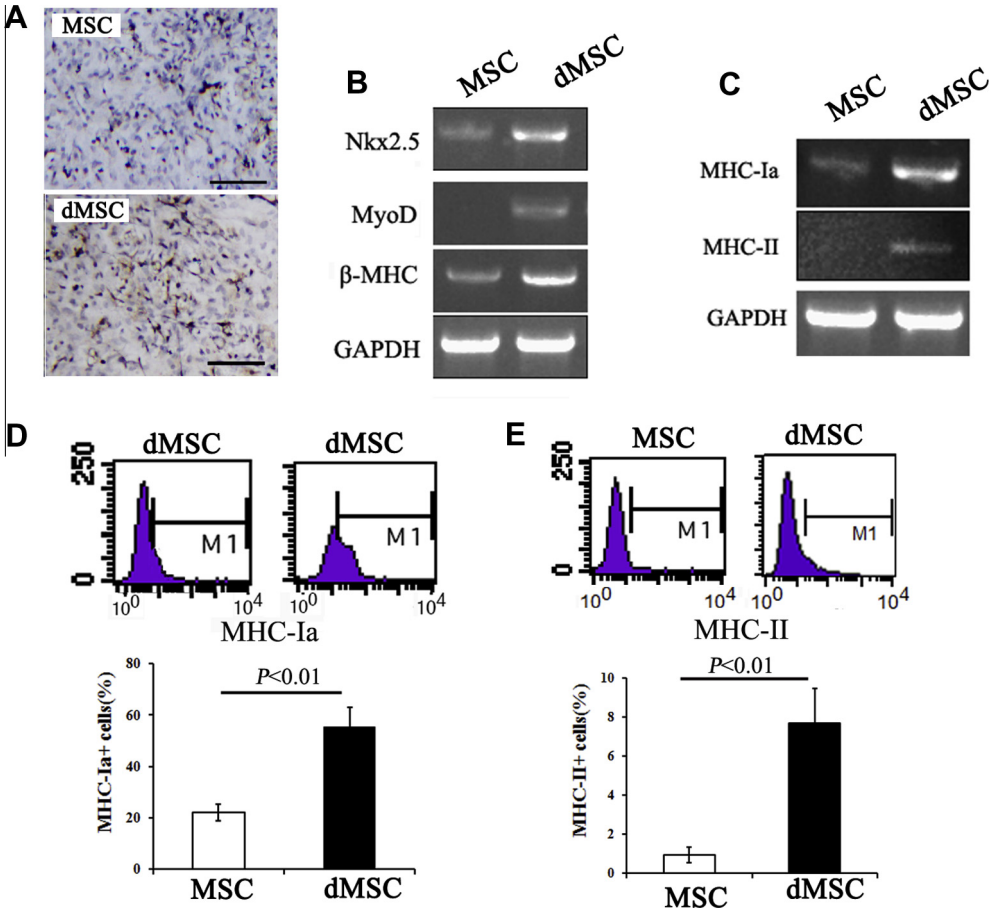

Fig. 2. Cardiac differentiation and immune antigen expression of mouse MSCs *in vitro*. A, immunohistochemistry against cTnT. The result demonstrated that 5-AZ treatment significantly induced the expression of cardiac protein in mouse MSCs (bar = 200 μm); B, RT-PCR revealed that the expression of cardiac genes in MSCs were significantly upregulated by 5-AZ treatment, providing additional evidence for induced cardiac differentiation of mouse MCSs by 5-AZ; C, RT-PCR revealed the immune antigen expression in differentiated MSCs was increased; D, quantitative analysis by flow cytometry showed that immune antigen expression (MHC-Ia and MHC-II) was much higher in differentiated MSCs than that in undifferentiated MSC. MSC: undifferentiated MSC or control MSC; dMSC: pre-differentiated MSC treated by 5-AZ.

imaging on MSCs demonstrated that the number of MSCs was linearly correlated with BLI signals ($R^2 = 0.992$), indicating that the reporter could be used to quantitatively track MSCs.

3.2. Cardiac differentiation and immune antigen expression of MSCs

During the culture after treatment with 5-AZ, the mological changes of MSCs could be observed. After two weeks, elongated cells of myotubular morphology were found in the culture. Immunostaining against cTnT demonstrated that cardiac-like cells expressing cTnT existed in the differentiated cells, while no such cells were found in the control cells (Fig. 2A). The results suggested that MSCs have differentiated toward cardiac lineage. RT-PCR also showed that cardiomyote genes were significantly increased in 5-AZ treated cells (Fig. 2B). To determine whether immune antigens were changed during 5-AZ induced differentiation, we assessed the expression of MHC class I (MHC-Ia) and MHC class II (MHC-II). As shown in Fig. 2B, the differentiated cells demonstrated a high expression of MHC-Ia and MHC-II, while none (MHC-II) or low (MHC-Ia) expression of immune antigens was detected in undifferentiated cells. Then, we performed quantitative analysis on the expression of immune antigens with flow cytometry. The results demonstrated that both MHC-Ia and MHC-II expression in differentiated MSCs were significantly higher than that in undifferentiated MSCs (Fig. 2D and E), providing further evidence for the increased immunogenicity of 5-AZ treated MSCs.

3.3. Bioluminescence imaging of transplanted cells in vivo

After surgery, mice receiving transplantation of MSCs or pre-differentiated MSCs were imaged longitudinally (1 day, 1 week, 2 weeks and 4 weeks). At day 1, we found that the BLI signals in mice receiving MSC transplantation were compatible with that receiving pre-differentiated MSCs, indicating that no significant difference has been made between the two groups during cell injection (Fig. 3). However, at the following detection (1 week), BLI signals in the two groups dramatically decreased compared with that at day 1, indicating that transplanted cells encountered mass death within 1 week following myocardial transplantation. This phenomenon was consistent with previous report [11]. In contrast, BLI signals from mice receiving MSC injection were much higher than that receiving pre-differentiated MSC injection ($P < 0.01$). At 2 weeks, BLI signals from mice receiving pre-differentiated MSC injection demonstrated a further dramatic decrease, no visible signals was observed (comparable to background as shown in Fig. 3). From 1 to 2 weeks, further decrease in BLI signals was also observed in mice receiving MSC injection, but it was much more attenuated, only a slight decrease was observed and significant BLI signals could be still detected. At 4 weeks, visible signals were still observed in MSC-injected mice, though it was much lower compared with that in the previous detection. The data suggested that pre-differentiated MSCs would be more rapidly excluded in allogeneic myocardium, consistent with the *in vitro* data that 5-AZ treated MSCs possessed a higher immunogenicity than undifferentiated ones.

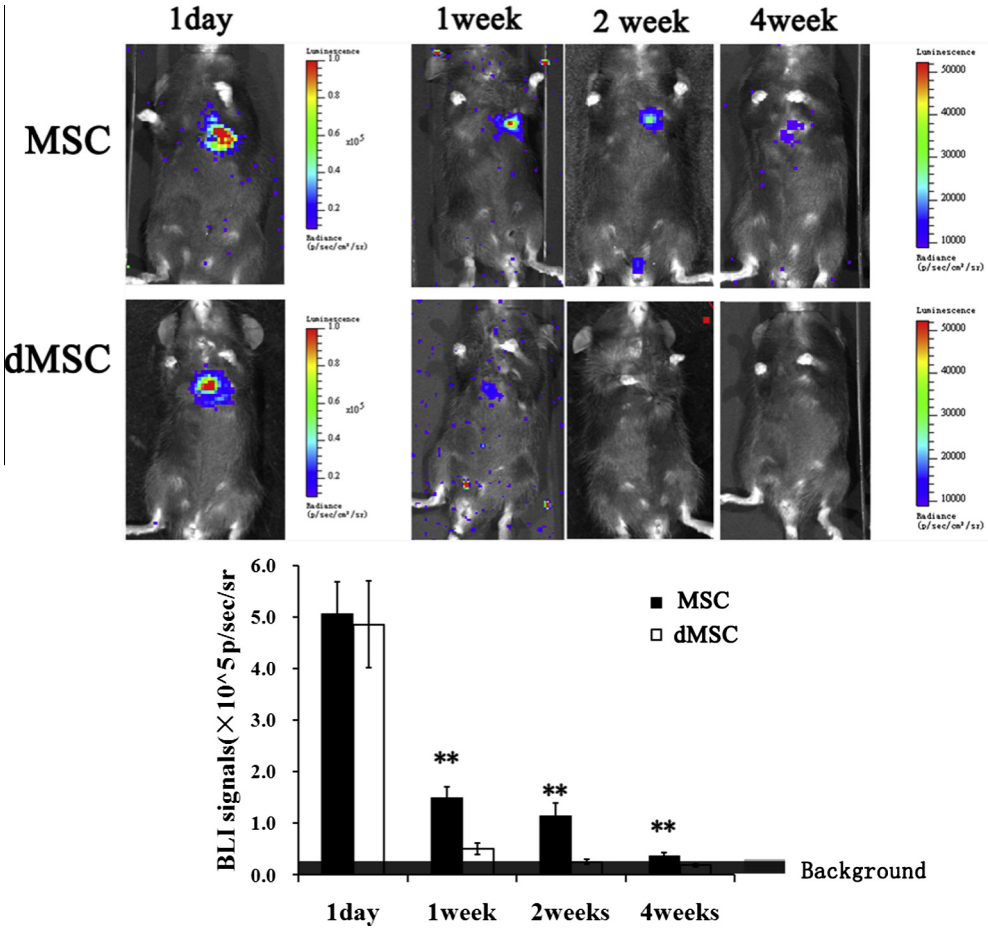

Fig. 3. Bioluminescence imaging of transplanted cells. Both BLI signals from mice receiving MSC and dMSC transplantation showed a progressive decrease with time, but the decrease in dMSC-transplanted mice was much more dramatic, indicating dMSCs were excluded much more rapidly from allogeneic hearts than MSCs. $^{**}P < 0.01$.

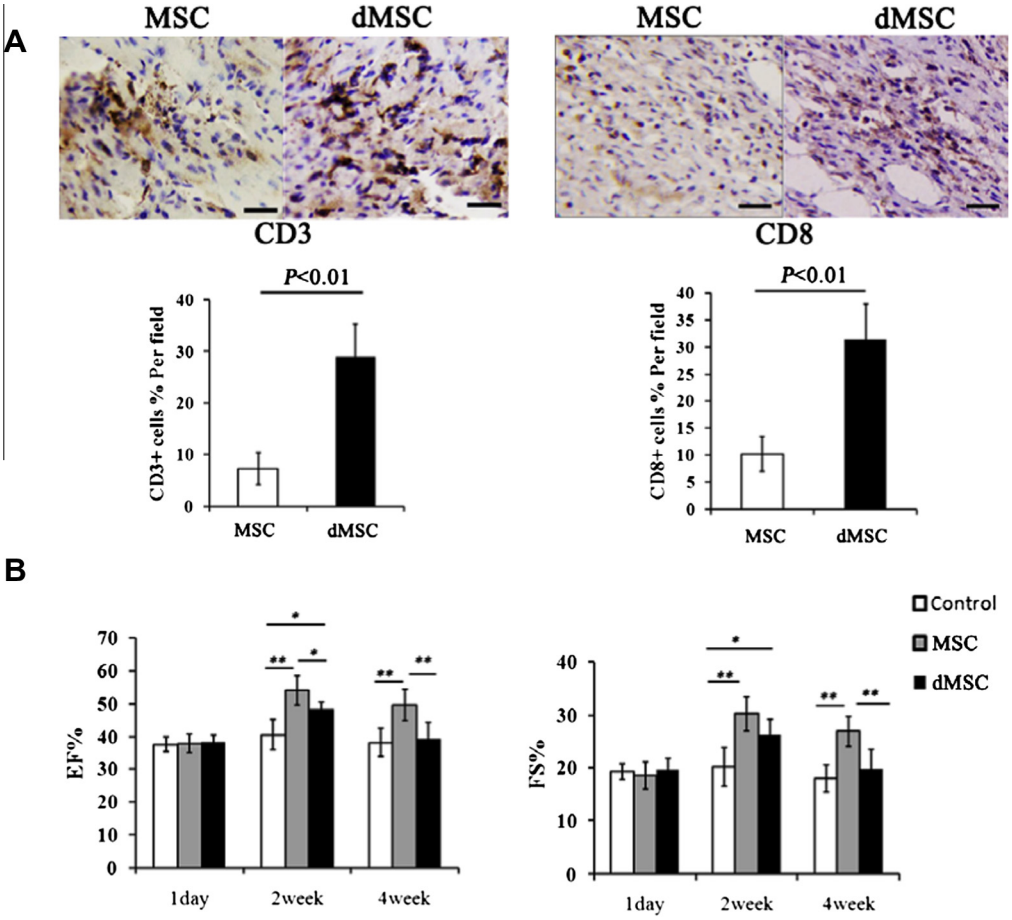

Fig. 4. Immune cell infiltration and cardiac function after cell transplantation. A, immune cell infiltration 1 week after cell transplantation.CD3 is a surface marker of T lymphocytes, while CD8 is a marker of cytotoxic T cells; B, cardiac functional parameters, LVEF and LVFS were measured at 1 day, 2 and 4 weeks after cell transplantation. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.4. Immune cell infiltration in vivo

To further confirm whether 5-AZ MSCs elicited a more severe immune response from allogeneic receipts, immunostaining against CD3 and CD8 was performed on the heart slices at 1 week after cell transplantation. CD3 is a surface marker of T lymphocytes, while CD8 is a marker of cytotoxic T cells.

In section from MSC-transplanted mice, only a slight infiltration of CD3+ and CD8+ cells was detected. In contrast, much more immune cells were observed in section from mice receiving 5-AZ treated-MSC transplantation. Quantitative analysis indicated that the immune cell infiltration elicited by 5-AZ treated-MSCs was significantly more severe than that by MSCs (Fig. 4A, $P < 0.01$).

3.5. Cardiac function

Echocardiography was performed to reveal the beneficial effects of pre-differentiated MSCs on ischemic hearts. At 1 day after surgery, LVEF and LVFS in mice receiving different treatment were comparable, indicating standardized LAD (Fig. 4B). At 2 weeks, both MSCs and pre-differentiated MSCs significantly improved cardiac function compared with control (MSC VS control: $P < 0.01$; pre-differentiated MSC VS control: $P < 0.05$). In comparison, MSCs were more effective in improvement of cardiac function (LVEF between MSC and pre-differentiated MSC: $P < 0.05$). This may be due to the mass exclusion of pre-differentiated MSCs from host myocardium. At 4 weeks, the protective effects of transplanted MSCs on heart function were still observed, but the effects produced by pre-differentiated MSCs have disappeared. Both LVEF and LVFS in MSC-transplanted mice were significantly higher than that in control and pre-differentiated MSC transplanted mice (both $P < 0.01$). The data suggested that both MSCs and pre-differentiated MSC produced beneficial effects on cardiac function of allogeneic ischemic hearts. However, due to the increased immunogenicity with differentiation, pre-differentiated MSCs would be rapidly excluded from allogeneic receipts, resulting in a much shorter term of the beneficial effects.

4. Discussion

Allogeneic MSCs are appealing sources for cardiomyoplasty in clinical due to their low expression of immune antigens. When transplantation of allogeneic MSCs comes into practice, it will be of great benefits for patients in that time-consuming procedure for isolation and expansion of autologous cells is not necessary. Instead, sufficient seeding cells could be stored for potential usage. More importantly, it will be easy to meet those patients whose cells are not suitable for transplantation, e.g., isolating MSCs from young donors for the elderly. In recent years, increasing more studies focused on the usage of allogeneic MSCs, but various results were acquired.

To enhance the capability of myocardial regeneration by MSCs, pre-differentiation with 5-AZ treatment has previously been adopted by different groups [12,16]. However, the efficacy of the strategy remains undefinite. In the study, we focused on the

survival and utility of allogeneic MSCs in ischemic myocardium and found that: (1) MSCs possessed a low immunogenicity that they could survive for more than 1 month after transplantation in allogeneic hearts; (2) 5-AZ treatment induced cardiac differentiation from MSCs, which would also increase the expression of their immune antigens; (3) transplantation of pre-differentiated MSCs elicits severe immune response from allogeneic recipients, resulting in the rapid exclusion of transplanted cells and a very short beneficial effect.

In previous studies, Huang et al. have demonstrated that differentiation would increase the immunogenicity of MSCs [8]. Though undifferentiated MSCs could survive in allogeneic hearts, they would be rapidly recognized by recipient immune system with time due to *in vivo* differentiation. However, Dai et al. have observed inconsistent results, that allogeneic MSCs survived in recipient myocardium for 6 months despite their differentiation [5]. In present study, we adopted a more accurate and non-invasive method, bioluminescence imaging, for MSC tracking. The method allowed for longitudinal detection and provided more quantitative information for cell survival. With the method, we obtained consistent results with Huang et al. [8]. We found that very few cells survived in allogeneic recipients at 4 weeks after transplantation as nearly no visible BLI signals were detected. However, significant improvement of cardiac function was still observed despite the exclusion, indicating cardiac protection by MSCs is independent of their integration with host myocardium. The results were also consistent with the previous report.

5-AZ treatment has been confirmed to enhance cardiac differentiation from MSCs for long time [4,25]. It was also adopted by different groups to enhance the efficacy of MSC for MI [12,16], considering that regenerated cardiomyocytes by MSCs may be increased. Actually, several studies have really observed improved protection of 5-AZ treated MSCs on MI in syngeneic recipients. However, most of these studies observed the short-term effects (1 month), whether 5-AZ treated MSCs could exert a long-term effects as undifferentiated MSCs was unclear. In the study, in an allogeneic MI model, we demonstrated that 5-AZ treated MSCs would be rapidly excluded due to their increased immunogenicity. Moreover, their beneficial effects on ischemic hearts would disappear within a month, inferior to undifferentiated MSCs. These data suggested that simple treatment by 5-AZ may not be so good an option for enhancing MSC-based myocardial repair.

In conclusion, we demonstrated that 5-AZ treatment increase the immunogenicity of MSCs and thus, 5-AZ treated MSCs will be rapidly recognized and excluded by allogeneic host immune system after transplantation. Consequently, only a short effect on MI would be produced by 5-AZ treated MSCs, worse than transplantation of undifferentiated MSCs.

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