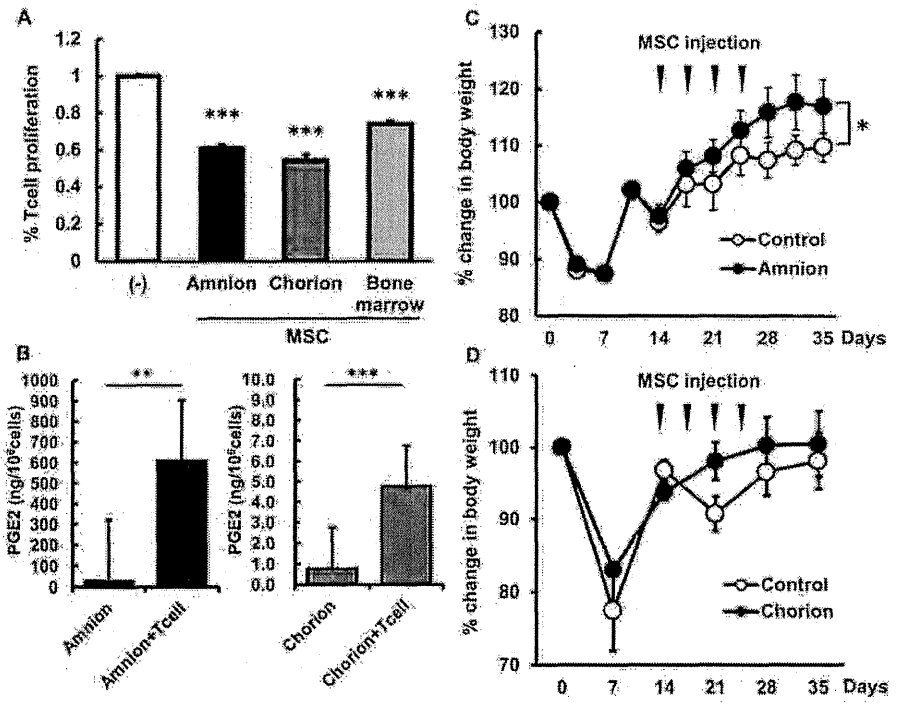

Figure 4. Immunosuppressive property of amnion and chorion MSCs. (A) Inhibition of human CD4+ T cell proliferation upon co-culture with human amnion, chorion, and bone marrow MSCs. (B) The concentration of PGE2 in FM-MSC-conditioned medium was measured by ELISA. Amnion MSCs secreted a significant amount of PGE2 compared with chorion MSCs. (C, D) Effect of human amnion (C) or chorion (D) MSC transplantation in a murine GVHD model. Treatment with amnion MSCs significantly reduced recipient weight loss in a mouse model of GVHD. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

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DR (Figure 1D), which satisfied the criteria for identifying MSCs [10]. In addition, amnion and chorion MSCs could differentiate into adipocytes and osteocytes, as demonstrated by positive Oil Red O and Alizarin Red S staining, respectively (Figure 1E and 1F).

Cytoprotective Effects of Amnion and Chorion MSCs on Endothelial Cells and Cardiomyocytes

To evaluate the cytoprotective effect of amnion and chorion MSCs, we examined cell viability and apoptosis of HUVECs and neonatal rat cardiomyocytes cultured under serum deprivation. In the MTS assay, cell viability of cardiomyocytes was significantly increased when cultured with conditioned medium obtained from amnion and chorion MSCs (absorbance value: serum-free control 0.331 ± 0.002, amnion MSCs 0.359 ± 0.006; $p<0.001$, and chorion MSCs 0.355 ± 0.004; $p<0.01$ vs. control) (Figure 2B). Cell viability of HUVECs also increased when cultured with chorion MSC-derived conditioned medium (serum-free control 0.263 ± 0.013, amnion MSCs 0.247 ± 0.014, and chorion MSCs 0.313 ± 0.012; $p<0.05$ vs. control) (Figure 2A). Similarly, conditioned medium obtained from chorion MSCs significantly decreased the caspase-3 activity of HUVECs (absorbance value: serum-free control 0.201 ± 0.006 vs. chorion MSCs 0.159 ± 0.004; $p<0.001$) and cardiomyocytes (control 0.106 ± 0.007 vs. chorion MSCs 0.079 ± 0.004; $p<0.05$) (Figure 2C, D). Amnion MSC-derived conditioned medium also showed a tendency to decrease the caspase-3 activity of these cells, but without statistical significance.

Secretion of Growth Factors from Cultured Amnion- and Chorion-derived MSCs

To investigate the secretion of major growth factors from MSCs, we performed ELISA of HGF, IGF-1, bFGF, and VEGF. The differences in the cellular expression profile of the growth factors were observed in these FM-derived MSCs (Figure 2E–H). Among these growth factors, amnion MSCs secreted significant amounts of HGF (1217.2 ± 80.2 pg/10^6 cells; $p<0.001$ vs. chorion-MSC) and bFGF (137.2 ± 18.5 pg/10^6 cells; $p<0.05$ vs. chorion-MSC) compared with chorion MSCs (HGF: 932.5 ± 85.3 pg/10^6 cells, bFGF: 93.6 ± 8.1 pg/10^6 cells) (Figure 2E, G). There was no significant difference between amnion and chorion MSCs in the level of secreted IGF-1 (88.8 ± 53.4 pg/10^6 cells and 205 ± 77.0 pg/10^6 cells, respectively) and VEGF (46.1 ± 12.3 pg/10^6 cells and 60.7 ± 5.3 pg/10^6 cells, respectively) (Figure 2F, H).

Augmentation of Angiogenesis in the Ischemic Hindlimb after Human FM-MSC Transplantation

Analysis of LDPI revealed that accelerated limb perfusion was observed in the amnion and chorion MSC-transplanted groups (Figure 3A). The LDPI index was significantly higher in the amnion and chorion MSC groups (amnion MSCs: 0.85 ± 0.07; $p<0.01$, chorion MSCs: 0.83 ± 0.05; $p<0.01$) than in the control group (0.56 ± 0.07) 5 days after transplantation (Figure 3B). At 7 days after transplantation, there was no difference between the treated and control groups.

Immunostaining with the endothelial marker CD31 showed significant augmentation of capillaries in the amnion and chorion

MSC-treated groups compared with the control group (Figure 3E). The capillaries-to-muscle-fiber ratio of ischemic muscle at day 5 after transplantation was significantly increased in the amnion and chorion MSC groups (amnion MSCs: 1.53±0.03/muscle fiber; $p<0.001$, chorion MSCs: 1.51±0.04/muscle fiber; $p<0.001$) compared with the control group (1.05±0.06/muscle fiber; Figure 3F). At day7, the capillaries-to-muscle-fiber ratio of ischemic muscle was also increased in the amnion or chorion MSC-transplanted mice (amnion MSCs: 1.67±0.17/muscle fiber, chorion MSCs: 1.43±0.09/muscle fiber) compared to the control mice (1.36±0.11/muscle fiber).

Immunosuppressive Property of Human FM-MSCs

Although the number of T cells was markedly increased under proliferating conditions of human CD4+ T cells stimulated with anti-CD3 and -CD28 antibodies, the increase was significantly suppressed when co-cultured with amnion-, chorion-, or bone marrow-derived MSCs (61.1±1.8%, 54.6±3.0%, 74.0±2.1%, respectively. $p<0.001$ vs. control) (Figure 4A).

PGE2 is a well-known immune modulator in bone marrow MSCs [13] and we confirmed that amnion MSCs in culture secreted a significant amount of PGE2 (29.7±7.8 ng/10^6 cells), particularly when co-cultured with human CD4+ T cells (613.1±139.9 ng/10^6 cells; $p<0.01$ vs. amnion MSCs) (Figure 4B). In chorion MSCs, however, the concentration of PGE2 was relatively low (0.77±0.13 ng/10^6 cells) but significantly increased in co-culture with CD4+ T cells (4.76±0.47 ng/10^6 cells; $p<0.001$ vs. chorion MSCs). The experiments were repeated with two or three independent MSC/CD4+ T cell donor pairs and the data are presented as the measured mean levels.

In addition, to evaluate the potential of FM-MSCs to suppress acute GVHD, mice underwent allogeneic hematopoietic stem cell transplantation and treatment with human FM-MSCs. As shown in Figure 4C, the loss in body weight of recipient mice after allogeneic hematopoietic stem cell transplantation was significantly reduced with concomitant transplantation of human amnion-derived MSCs. In human chorion MSC-transplanted group, however, no significant changes in body weight was observed during the observation period (Figure 4D).

Discussion

Human MSCs derived from bone marrow or adipose tissue exert a regenerative effect in animal models and human patients [14]. In addition, several reports have described the therapeutic potential of transplanted cells derived from the appendages of the fetus, including amniotic epithelium cells [15], and amniotic fluid- [16], amnion-, and chorion-derived MSCs [17,18]. We have previously demonstrated the therapeutic potential of rat FM-MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Recent studies including ours also revealed the angiogenic and immunosuppressive property of human fetal appendage-derived MSCs [14,18–20], but comparative studies of the therapeutic effects among these MSCs are lacking. Therefore, in this study, we examined the differences in the cellular function and therapeutic properties between human FM-derived amnion and chorion MSCs.

It is known that MSCs exert their regenerative effects through differentiation into specific cell types, but recent studies suggest that their ability to stimulate regenerative effects is mainly induced via paracrine effects [3,4,8,21]. This theory is substantiated by several reports that MSCs secrete various growth factors and cytokines including VEGF, IGF-1, HGF, adrenomedullin (AM), and PGE2 [3–5,8,21,22]. In this study, we first confirmed that chorion MSCs as well as amnion MSCs secreted significant amount of these soluble factors, which would contribute to accelerating regenerative effects. Compared with chorion MSCs, amnion MSCs secreted significantly larger amounts of HGF and bFGF. However, amnion MSCs secreted less IGF-1 compared to chorion MSCs. We assume that these differences in the cytokine expression profile might reflect the angiogenic and cytoprotective properties of amnion and chorion MSCs, as we observed difference in the effect on endothelial cells and cardiomyocytes in our conditioned-medium analysis. However, the actual function of amnion or chorion MSC-derived cytokines should be further investigated *in vivo* because both human amnion and chorion MSC transplantation similarly induced angiogenesis in the hindlimb ischemia model.

Previous reports have shown that PGE2 is a major modulator of the MSC-induced anti-inflammatory response [13]. In this study, a noteworthy finding was a distinctly high concentration of PGE2 in amnion MSCs in comparison with chorion MSCs, particularly when co-cultured with CD4+ T cells. Because of their high PGE2 production, human amnion MSCs might be a better cell source from an immunosuppressive point of view. In fact, we proved for the first time that human amnion MSCs, but not chorion MSCs, improved the pathological situation of an acute GVHD model. Because our previous study demonstrated that human amnion MSCs markedly inhibited differentiation as well as proliferation of Th1/Th17 cells [6], human amnion MSCs could effectively suppress Th1/Th17 immunity and improve outcome in GVHD.

The merit of using FMs lies in that they are free form ethical concern and that a large number of MSCs can be obtained considering the size of FM. As more than one or ten million MSCs per gram of the amnion or chorion could be obtained, more than 10^9 or 10^{10} MSCs could theoretically be obtained at passage 3 within one month, respectively. Now we are planning to initiate clinical studies with human amnion MSCs in acute GVHD and Crohn's disease, and we need more than 10^{10} MSCs for the treatment of one patient. We are convinced that human FM-MSCs are an attractive source for cell therapy because of their easy availability compared with other somatic, embryonic stem, and iPS cells.

In conclusion, both amnion and chorion MSCs have angiogenic, cytoprotective, and immunomodulatory effects. Because of high PGE2 production and immunosuppressive properties, human amnion MSCs have the advantage for the treatment of immune-related diseases. In addition, since a large number of MSCs could be obtained from FMs, human amnion and chorion MSCs would be a useful cell source for regenerative medicine.

Author Contributions

Conceived and designed the experiments: KY AT TS HO JY MHS KK TI. Performed the experiments: KY KH MO SI SO HT KO SK JY TI. Analyzed the data: KY KH MO TI. Contributed reagents/materials/analysis tools: KY KH MO TI. Wrote the paper: KY KH MO TI.

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Allogeneic Transplantation of Fetal Membrane–Derived Mesenchymal Stem Cell Sheets Increases Neovascularization and Improves Cardiac Function after Myocardial Infarction in Rats

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Background. Mesenchymal stem cell (MSC) transplantation has been pursued as a new method to repair damaged myocardium. We focused on the fetal membrane (FM) as an alternative source to bone marrow (BM)–derived MSCs. In this study, we investigated whether transplantation of allogeneic FM-MSC sheets could attenuate myocardial dysfunction in a rat chronic myocardial infarction (MI) model.

Methods. Sheets of allogeneic FM-MSC or autologous BM-MSC were transplanted into the scarred myocardium 4 weeks after coronary ligation.

Results. Four weeks after transplantation, both allogeneic FM-MSC and autologous BM-MSC sheets had significantly improved cardiac function and reduced myocardial fibrosis compared with the untreated MI group. In both MSC sheet-transplanted groups, the peri-infarct regional capillary density was increased. Some engrafted MSCs formed vascular structures and were positive for lectin I and α-smooth muscle actin. The numbers of engrafted cells and differentiated cells were very low after both types of MSC sheet transplantation. $CD3^+$ T cells did not increase in the transplantation site, but $CD163^+$ M2 macrophages increased in the groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Conclusions. Transplantation of allogeneic FM-MSC or autologous BM-MSC sheets attenuated myocardial dysfunction in a rat MI model to a similar degree. The engraftment rate of transplanted cells and immune cell infiltration into the transplanted area did not differ between the two types of MSC transplants. M2 macrophage induction has possible involvement in the therapeutic effects of MSC transplantation. Allogeneic FM-MSC sheet transplantation might be a new therapeutic strategy after MI.

Keywords: Fetal membrane, Mesenchymal stem cells, Cell sheet, Myocardial infarction, Allogeneic transplantation.
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Myocardial infarction (MI) causes loss of cardiac tissue and impairment of left ventricular function. Recent reports suggest that mesenchymal stem cells (MSCs) are a

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valuable cell source for cell therapy after MI and that bone marrow (BM) represents a major source of MSCs. Several clinical trials of autologous BM-MSC transplantation for MI have reported therapeutic success (*1–3*).

BM harvest is a surgical procedure that requires general anesthesia or sedation, and both the proliferative potential and the differentiation capacity of MSCs seem to decrease in older donors (*4, 5*). In addition, BM procurement procedures in humans may yield low numbers of MSCs after cell processing. To address this issue, we focused on the fetal membrane (FM) of the placenta, which is generally discarded as medical waste after delivery, as an alternative source of autologous MSCs. Several studies have reported that the human FM contains multipotent cells similar to BM-MSCs and that these cells are easy to expand (*6, 7*). We demonstrated previously that the allogeneic transplantation of FM-MSCs did not elicit any lymphocyte proliferative response despite their allogeneic origin and induced therapeutic effects in a rat model of hind-limb ischemia and acute myocarditis (*8, 9*).

In some types of MSC transplantation, dissociated MSCs are injected into the myocardium to induce cardiac regeneration. However, it is difficult to reconstruct sufficient cardiac mass in the thinned scar area after MI. Imanishi et al. (*10*) reported that approximately 90% of cells injected into the myocardium are lost within 1 day. Okano et al. recently developed cell sheets using temperature-responsive culture dishes (*11–14*). These cell sheets allow for cell-to-cell connections and maintenance of adhesion proteins. In a rat MI model, the engraftment rate of transplanted cells was higher after transplantation of cell sheets compared with intramyocardial transplantation of dissociated cells (*15, 16*). These results suggest that transplantation of allogeneic FM-MSC sheets may be a new strategy for the treatment of heart failure.

In this study, we designed a set of experiments with the following aims: (i) to compare the therapeutic effects of transplantation of allogeneic FM-MSC sheets and autologous BM-MSC sheets in a rat chronic MI model, (ii) to investigate the engraftment and differentiation of transplanted MSCs, and (iii) to investigate whether transplanted allogeneic FM-MSC sheets evade immune rejection.

RESULTS

Preparation and Transplantation of Two-Layered MSC Sheets

FM-MSCs derived from green fluorescent protein (GFP)-transgenic Sprague–Dawley rats (3.3×10^6 cells) or BM-MSCs derived from GFP-transgenic Lewis rats (3.3×10^6 cells) were cultured in temperature-responsive 35-mm dishes for 1 day. When the culture temperature was decreased from 37°C to 20°C, both types of MSC sheets detached spontaneously and floated into the culture medium as a monolayer MSC sheet that could be stacked into two-layer constructs (Fig. 1A, C, and D). We transplanted two-layered FM-MSC sheets or BM-MSC sheets over the anterior wall of the heart, including the infarcted area, and then attached them to the heart surface (Fig. 1B).

Engraftment of Transplanted Allogeneic FM-MSC and Autologous BM-MSC Sheets in Infarcted Hearts

One day and 1 and 2 weeks after transplantation, GFP-positive allogeneic FM-MSCs and autologous BM-MSCs were present as sheets on the infarcted area of the anterior wall (n=3 in each group). GFP-positive allogeneic FM-MSCs and autologous BM-MSCs were observed in the anterior infarcted area 3 and 4 weeks after transplantation (n=4 in each group) (Fig. 1E). However, semiquantitative analysis demonstrated that the engraftment rate decreased with time in both MSC sheet-transplanted groups (Fig. 1F). The engraftment rate did not differ significantly between the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Improvement in Cardiac Function after Transplantation of Allogeneic FM-MSC and Autologous BM-MSC Sheets

Heart failure developed 4 weeks after coronary ligation, as indicated by deterioration of left ventricular function and thinning of the infarct wall. The ejection fraction, fractional shortening, anterior wall thickness, posterior wall thickness, left ventricular diastolic dimension, and left ventricular systolic dimension measurements at baseline did not differ significantly between the three MI groups. One of the 10 rats in the untreated MI group died on day 53 after coronary ligation; no rats died in the MI groups transplanted with allogeneic FM-MSC or autologous BM-MSC.

Hemodynamic analysis revealed significant improvements in the MI group transplanted with allogeneic FM-MSC compared with the untreated MI group for the left ventricular systolic pressure, maximum dP/dt, and minimum dP/dt ($P<0.05$; n=10 in each group) (Fig. 2B,C; see **Table S1, SDC,** http://links.lww.com/TP/A849).

Echocardiographic analysis revealed significant improvements in ejection fraction, fractional shortening, and left ventricular systolic dimension ($P<0.05$ for each) in the MI group transplanted with allogeneic FM-MSC compared with the untreated MI group. Anterior wall thickness was also significantly greater in the MI group transplanted with allogeneic FM-MSC than in the untreated MI group (Fig. 2D,E; see **Table S2, SDC,** http://links.lww.com/TP/A849).

The hemodynamic and echocardiographic parameters did not differ significantly between the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Reduction in Myocardial Fibrosis after Transplantation of Allogeneic FM-MSC and Autologous BM-MSC Sheets

Eight weeks after coronary ligation, Masson's trichrome staining of the myocardium from the untreated MI group demonstrated prominent and diffuse interstitial fibrosis in the anterior scar area. This was attenuated markedly in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC (Fig. 3A). Quantitative assessment of myocardial fibrosis of the left heart showed that the fraction of Masson's trichrome-stained collagen volume was significantly smaller in the MI groups transplanted with allogeneic FM-MSC or autologous BM-MSC than in the untreated MI group ($P<0.05$; n=10 in each group) (Fig. 3B).

Angiogenesis and Differentiation of Transplanted Allogeneic FM-MSC and Autologous BM-MSC Sheets in Infarcted Hearts

Four weeks after transplantation, vascularization was assessed by lectin I staining and was observed in the allogeneic FM-MSC–transplanted MI group and the autologous

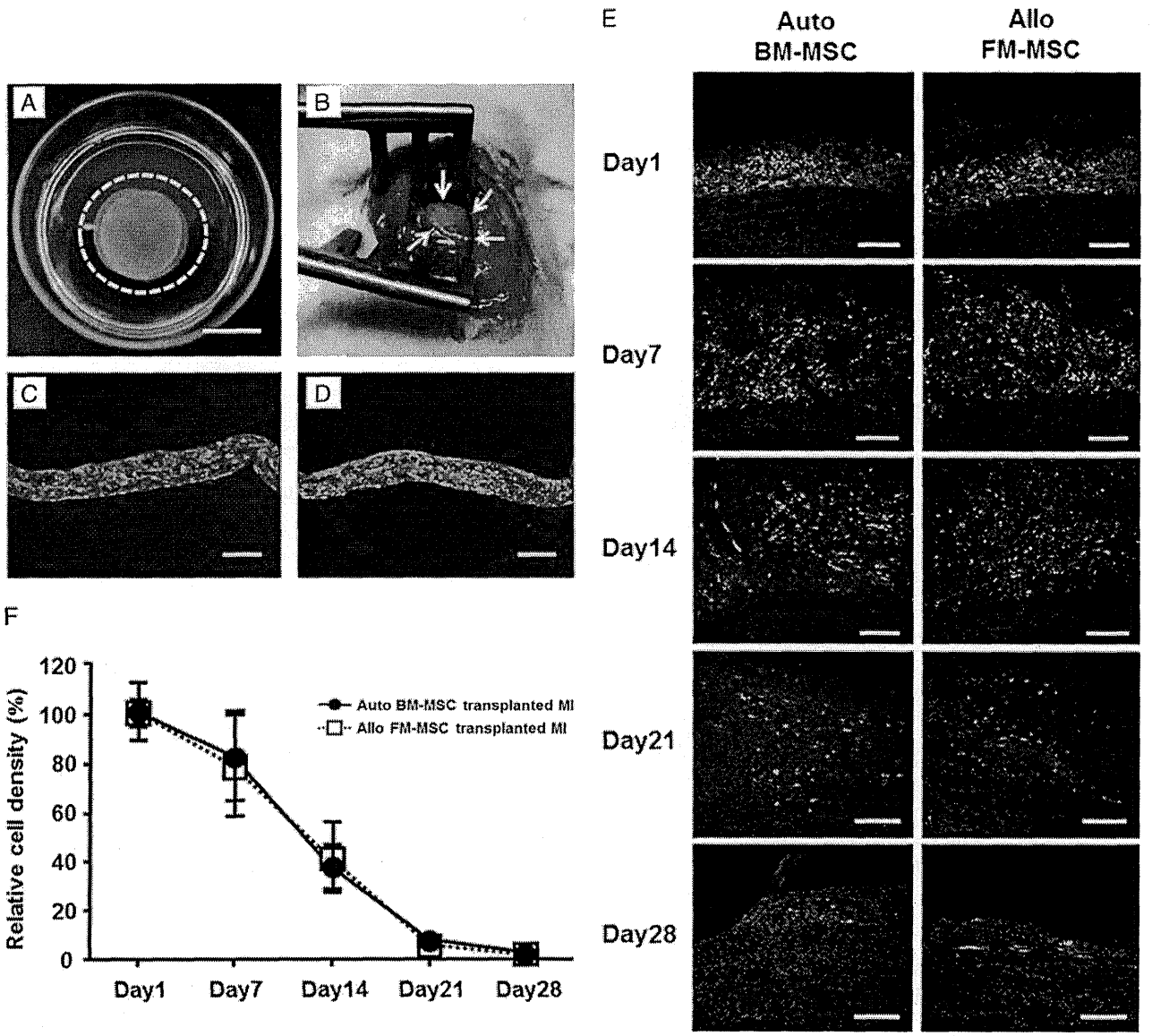

FIGURE 1. Stacked MSC sheets and their transplantation into infarcted hearts. A, two MSC sheets harvested from temperature-responsive culture surfaces were stacked successfully, producing a two-layer construct. Scale bar, 10 mm. B, two-layered MSC sheets were transplanted over the anterior wall of the infarcted heart and formed a stable attachment to the heart surface (arrows). C, cross-sectional staining of a GFP-expressing two-layered FM-MSC sheet. Scale bar, 100 μm. D, cross-sectional staining of a GFP-expressing two-layered BM-MSC sheet. Scale bar, 100 μm. E, allogeneic FM-MSCs and autologous BM-MSCs were present over the area surrounding the scar on days 1, 7, 14, 21, and 28. Scale bar, 100 μm. F, semiquantitative analysis showed that the engraftment rate of cells decreased with time in both groups transplanted with MSC sheets (days 1, 7, and 14, n=3 in each group; days 21 and 28, n=4 in each group). Data are expressed as mean±SE.

BM-MSC–transplanted MI group (Fig. 4B). Quantitative analysis showed increased capillary density in the infarcted area in both MSC-transplanted groups compared with the untreated MI group (n=6 in each group) (Fig. 4C). The capillary density in the peri-infarct area was similar in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC; both values were significantly higher than in the untreated MI group ($P<0.05$; n=6 in each group) (Fig. 4D).

GFP-positive FM-MSCs and BM-MSCs were observed in the peri-infarct area of the anterior wall, but GFP–lectin I/α-smooth muscle actin (αSMA) double-positive cells were not observed 1 day or 1 week after transplantation (data not shown). Two weeks after transplantation, engrafted GFP-positive FM-MSCs and BM-MSCs formed vascular structures and were positive for lectin I and αSMA (Fig. 4E,F). The GFP–lectin I/αSMA double-positive cells comprised less than 1% of the engrafted cells.

Immune Responses to Transplanted Allogeneic FM-MSCs and Autologous BM-MSCs in Infarcted Hearts

To compare the host immune responses to transplanted allogeneic FM-MSCs and autologous BM-MSCs, we performed immunohistochemical staining for CD3 (T cells) and CD68

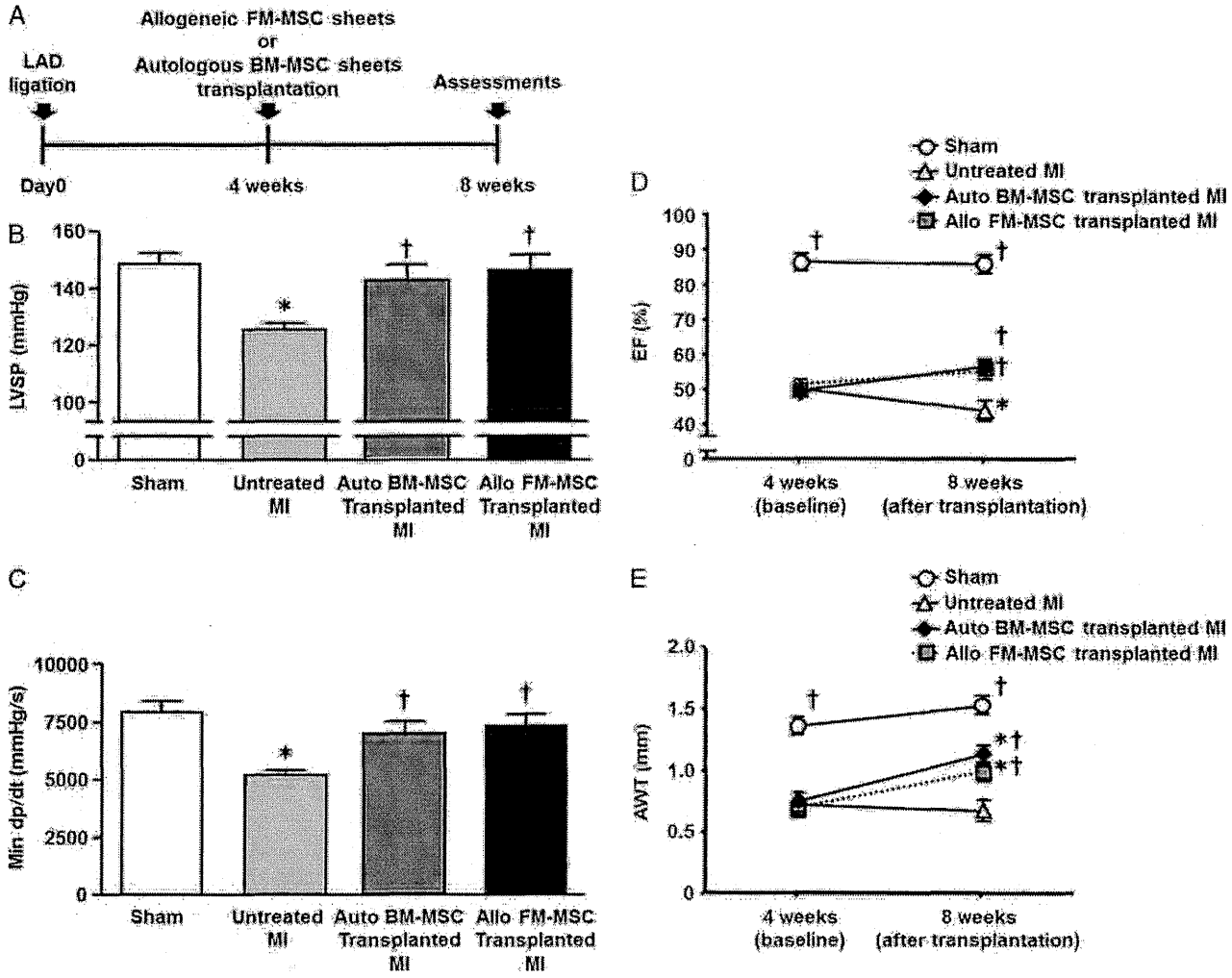

FIGURE 2. Effects of transplantation of allogeneic FM-MSC and autologous BM-MSC sheets on hemodynamic and echocardiographic parameters after MI. A, study flowchart. B and C, four weeks after transplantation, left ventricular systolic pressure and minimum d*P*/d*t* had improved significantly in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC compared with the untreated MI group (n=10 in each group). Data are expressed as mean±SE. **P*<0.05 vs. sham group; †*P*<0.05 vs. untreated MI group. D and E, four weeks after transplantation, the ejection fraction and anterior wall thickness in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC had improved significantly compared with the untreated MI group (n=10 in each group). Data are expressed as mean±SE. **P*<0.05 vs. baseline; †*P*<0.05 vs. time-matched untreated MI group. AWT, anterior wall thickness; EF, ejection fraction; LVSP, left ventricular systolic pressure.

(monocytes and macrophages) in sections of MSC-transplanted infarcted hearts 4 weeks after transplantation. Compared with the sham group, the numbers of $CD3^+$ and $CD68^+$ cells in the infarcted and peri-infarct areas were increased in the untreated MI group and in the MI groups transplanted with allogeneic FM-MSC or autologous BM-MSC (Fig. 5A,D). Quantitative analysis demonstrated no significant differences in $CD3^+$ cell infiltration between the MI groups that were untreated or transplanted with allogeneic FM-MSC and autologous BM-MSC (n=8 in each group) (Fig. 5C).

In the infarcted areas, there were no differences in the number of $CD68^+$ cells between the three MI groups, untreated or transplanted with allogeneic FM-MSC or autologous BM-MSC (n=8 in each group). By contrast, the number of $CD68^+$ cells in the peri-infarct area was significantly higher in both MSC-transplanted MI groups than in the untreated MI group (*P*<0.05 vs. untreated MI group; n=8 in each group) (Fig. 5E,F). The intensity of CD3 and CD68 staining did not differ between the two MSC-transplanted groups. $CD163^+$ cells were observed in the serial sections of the sites infiltrated by $CD68^+$ cells from all three MI groups (see **Figure S2b, SDC,** http://links.lww.com/TP/A849).

DISCUSSION

In the present study, we have demonstrated five points. First, transplantation of allogeneic FM-MSC sheets and autologous BM-MSC sheets improved cardiac function and prevented ventricular remodeling in a rat model of MI to a similar degree. Second, massive angiogenesis was observed in the areas transplanted with allogeneic FM-MSC sheets and autologous BM-MSC sheets but was not observed in the area

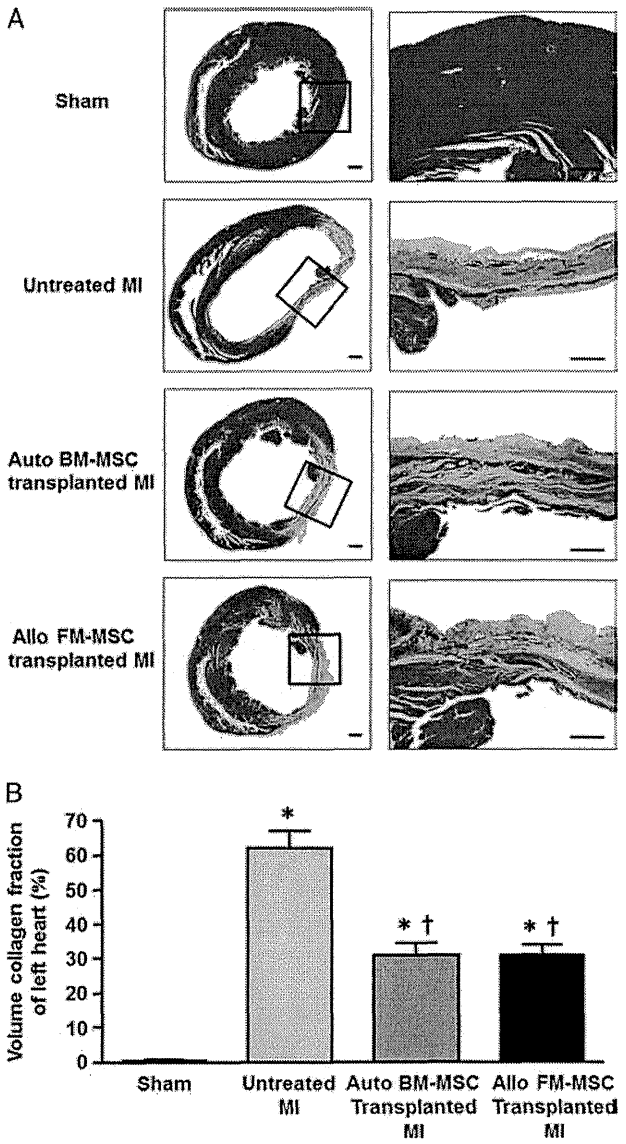

FIGURE 3. Masson's trichrome staining of heart cross-sections at the left ventricular papillary muscle level from MI rats transplanted with allogeneic FM-MSC and autologous BM-MSC sheets. A, four weeks after transplantation, the myocardial fibrosis area was smaller in the MI groups transplanted with FM-MSC and autologous BM-MSC than in the untreated MI group. Right row shows a higher resolution of the image in the black box in the respective left row. Scale bar, 1 mm (left row) and 500 μm (right row). B, quantitative analysis demonstrated that the fibrosis area was significantly smaller in the MI groups transplanted with FM-MSC and autologous BM-MSC compared with the untreated MI group (n=10 in each group). Data are expressed as mean±SE. *P<0.05 vs. sham group; †P<0.05 vs. untreated MI group.

of the infarcted myocardium. Third, transplanted allogeneic FM-MSCs engrafted in the infarcted myocardium from 1 day to 4 weeks after transplantation, but the number of engrafted cells decreased markedly with time. Fourth, some of the engrafted FM-MSCs were positive for lectin I or αSMA, but these cells comprised less than 1% of the engrafted cells. Fifth, the engraftment rate and host immune cell responses did not differ between groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Several studies have reported that transplantation of autologous BM-MSC improves cardiac function in ischemic heart disease (*17–20*). However, there are several limitations when using autologous BM-MSCs for clinical applications, including the invasiveness of the harvesting procedure, inadequate cell numbers, and donor site morbidity (*21*). We have reported that allogeneic FM-MSCs are an alternative to autologous BM-MSCs (*8, 9*). Although allogeneic, transplanted FM-MSCs exerted therapeutic effects in experimental rat models of hind-limb ischemia and acute myocarditis and did not elicit alloreactive lymphocyte proliferation. In this study, we showed a significant improvement in cardiac function and a reduction in myocardial fibrosis in rats with chronic MI that were transplanted with allogeneic FM-MSC sheets or with autologous BM-MSC sheets. The FM contains large quantities of MSCs, and their use is considered to present few ethical concerns; thus, FM-MSCs can provide a cell source for regenerative medicine (*22, 23*).

The mechanisms underlying the effectiveness of MSC therapy in treating ischemic heart failure may involve both the differentiation of transplanted MSCs into vascular cells and cardiomyocytes and the secretion of several growth factors by transplanted cells (paracrine effects). Two to 4 weeks after transplantation, some of the engrafted FM-MSCs and BM-MSCs stained positively for lectin I and participated in vessel formation. Staining for αSMA revealed that both types of MSCs differentiated into vascular smooth muscle cells, which play an important role in vessel maturation. A few engrafted MSCs may transdifferentiate in the vessel, but the number of such cells would be insufficient to be the main mechanism responsible for the therapeutic gain. We did not find desmin-positive or troponin T–positive engrafted allogeneic FM-MSCs or autologous BM-MSCs (data not shown). Earlier studies reported that transplanted MSCs differentiated into cardiomyocytes, vascular endothelial cells, and smooth muscle cells (*24–26*), but more recent studies have reported that transplanted MSCs appear to differentiate into these cells at a very low frequency (*27–30*).

After the discovery of the paracrine effect of MSCs, many studies have confirmed that the success of stem cell therapy for heart failure depends on this mechanism mainly by the promotion of angiogenesis, myocardial protection, and immune regulation (*31, 32*). In our previous study, transplanted FM-MSCs and BM-MSCs secreted angiogenic and cardioprotective cytokines, including vascular endothelial growth factor (VEGF) and hepatocyte growth factor, in the ischemic tissues (*8, 33*). These growth factors secreted from engrafted MSCs may help prevent ventricular remodeling. The response of the MSC sheets was similar, with large amounts of VEGF secreted into the culture media by FM-MSCs and BM-MSCs (see **Figure S1a, SDC,** http://links.lww.com/TP/A849). In both MI groups transplanted with MSC sheets, VEGF expression was up-regulated in the peri-infarct areas (see **Figure S1b, SDC,** http://links.lww.com/TP/A849). These results suggest that the therapeutic effects observed in our study may be attributable to the paracrine effects of transplanted FM-MSCs rather than to their differentiation into vascular endothelial cells and cardiomyocytes.

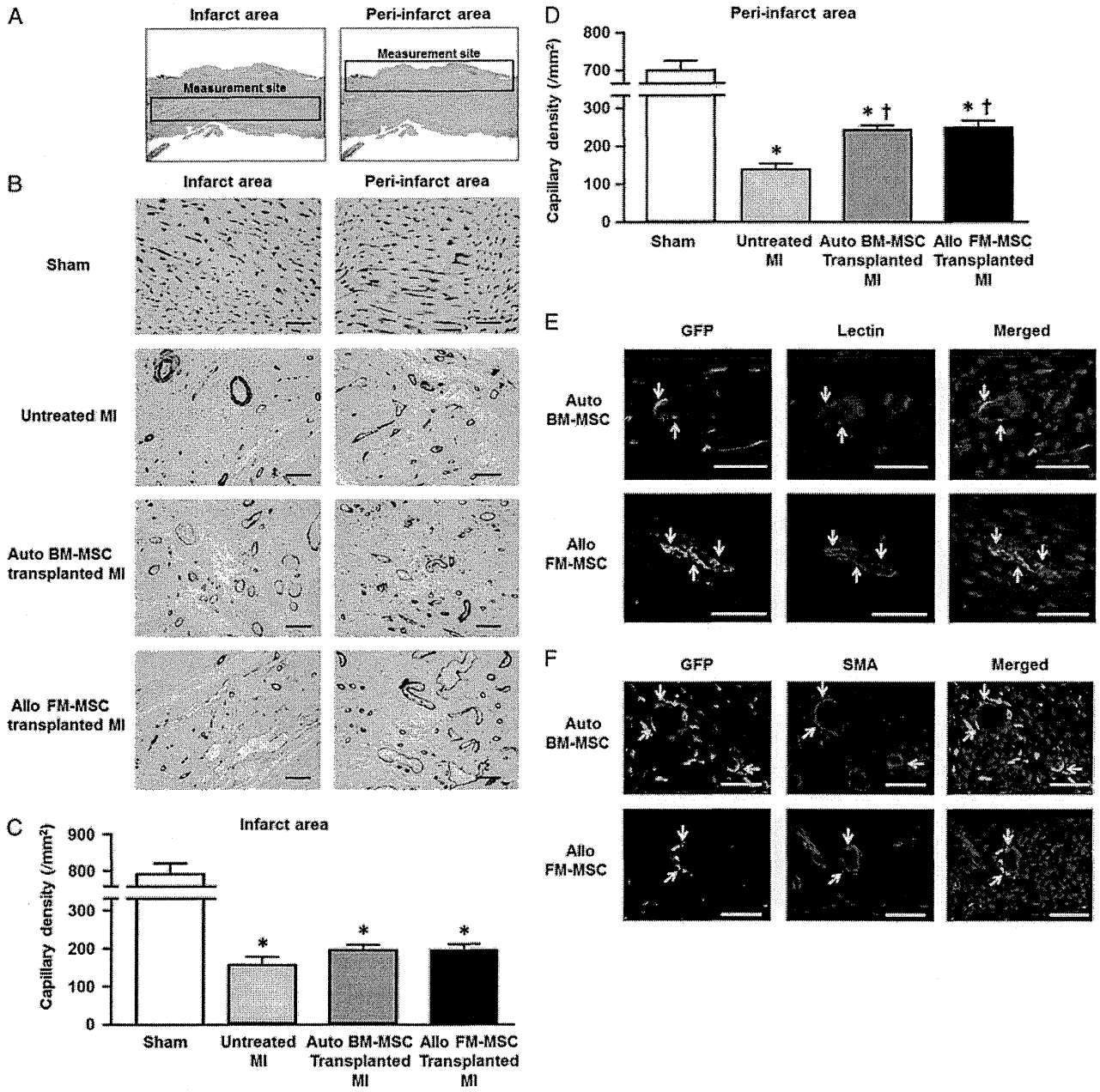

FIGURE 4. Vascularization and differentiation into vascular endothelial cells in the myocardial tissue grafted with allogeneic FM-MSC and autologous BM-MSC sheets. A, representative measurement section sites. B, four weeks after transplantation, the numbers of lectin I–positive capillaries were greater in the infarcted and peri-infarct areas in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC than in the untreated MI group. Quantitative analysis demonstrated that the capillary densities in the transplanted area were significantly higher in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC compared with the untreated MI group (infarcted area [C] and peri-infarct area [D]; n=10 in each group). E, two weeks after transplantation, GFP-expressing FM-MSCs and BM-MSCs were identified in a thick stratum on the epicardial side of the myocardium. Some allogeneic FM-MSCs and autologous BM-MSCs (green; white arrows) were positive for lectin I (red). F, some allogeneic FM-MSCs and autologous BM-MSCs (green; white arrows) were positive for αSMA (red). Nuclei are stained with TOPRO3 (blue). Scale bar, 50 μm. Data are expressed as mean±SE. *P<0.05 vs. sham group; †P<0.05 vs. untreated MI group.

MSCs are positive for major histocompatibility complex (MHC) I and negative for MHC II and costimulatory factors such as CD40, CD80, and CD86, so are considered to be nonimmunogenic (*34, 35*). We reported previously that FM-MSCs did not express MHC class II and did not induce alloreactive T lymphocyte proliferation (*8*). In this study, immunohistochemical staining showed few infiltrating $CD3^+$ T cells in the areas transplanted with allogeneic

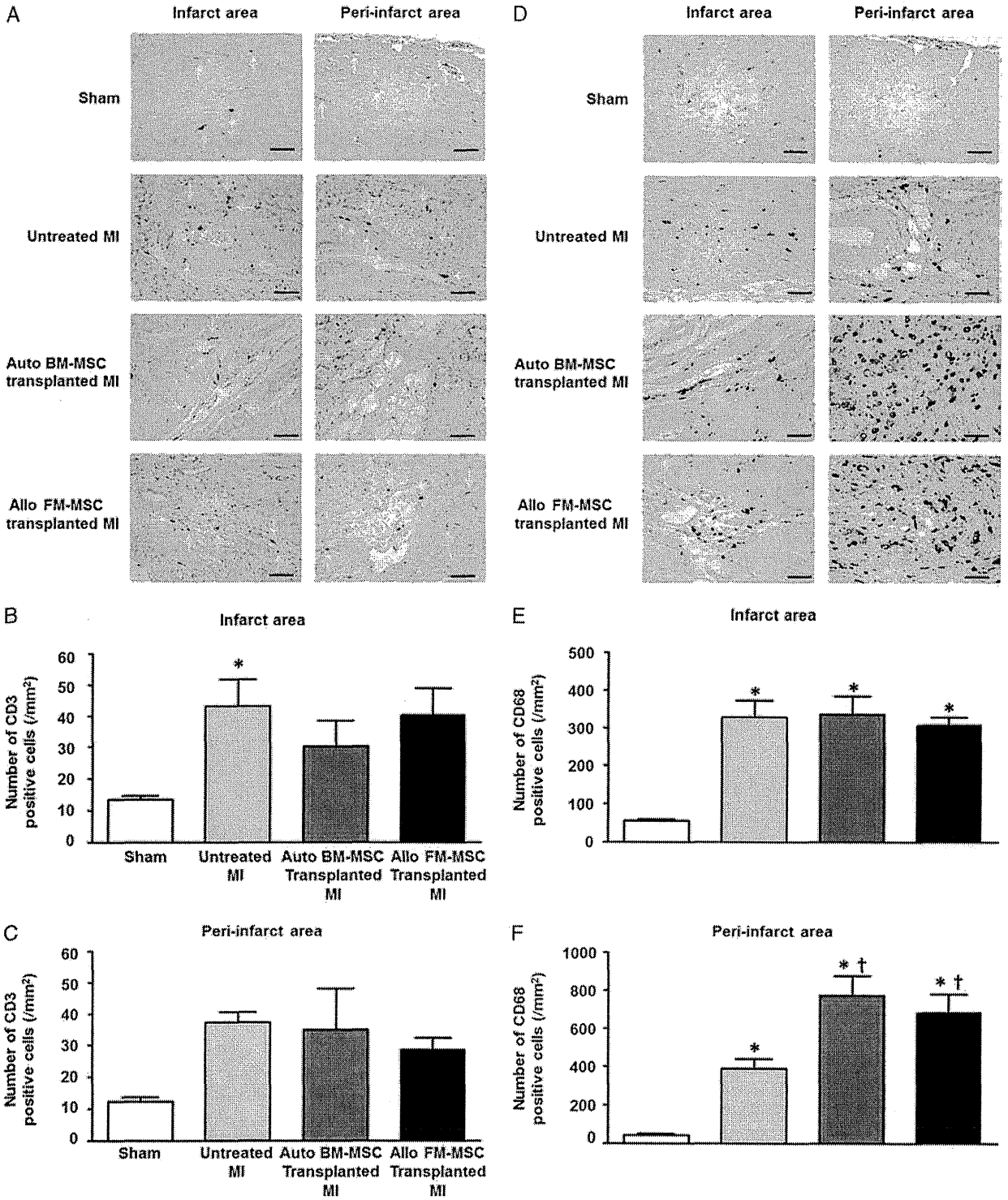

FIGURE 5. Immune responses in the myocardium transplanted with MSC sheets. A, in the infarcted and peri-infarct areas, the numbers of infiltrating $CD3^+$ cells did not differ between the untreated MI group and MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC (yellow arrows: $CD3^+$ cells). B and C, quantitative analysis of $CD3^+$ cells in the infarcted and peri-infarct areas showed no significant differences between the three MI groups (n=8 in each group). D, in the infarcted area, the number of infiltrating $CD68^+$ cells did not differ between the three MI groups, but marked $CD68^+$ cell infiltration was found in the peri-infarct area in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC. E and F, quantitative analysis of $CD68^+$ cells in the infarcted area showed no significant difference between the three MI groups. In the peri-infarct area, significantly more $CD68^+$ cells were found in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC than in the untreated MI group (n=8 in each group). Scale bar, 50 μm. Data are expressed as mean±SE. *P<0.05 vs. sham group; †P<0.05 vs. untreated MI group.

FM-MSCs and the infarcted areas at 4 weeks after transplantation, and the infiltrating T cells were almost all $CD8^+$ T cells (see **Figure S2a, SDC,** http://links.lww.com/TP/A849). However, there were no differences between the infarcted hearts transplanted with allogeneic FM-MSC sheets and untreated infarcted hearts in the number of infiltrating $CD3^+$ and $CD8^+$ T cells and the degree of $CD3^+$ T-cell infiltration, and the engraftment rate did not differ between allogeneic FM-MSC transplantation and autologous BM-MSC transplantation. Thus, this limited T-cell infiltration may have been caused by chronic myocardial inflammation. In addition, there were few infiltrating $CD45RA^+$ B cells in the allogeneic FM-MSC-transplanted areas (see **Figure S3, SDC,** http://links.lww.com/TP/A849). These results suggest that allogeneic FM-MSCs are unlikely to activate host immune responses. In contrast, massive $CD68^+$ macrophage/monocyte infiltration was observed in the areas transplanted with either type of MSCs. There are two conceivable reasons for this macrophage infiltration. First, the infiltrating macrophages may have phagocytosed apoptotic cells, because terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells were observed in areas transplanted with both types of MSCs (see **Figure S4, SDC,** http://links.lww.com/TP/A849). Second, there is a possibility that the macrophage infiltration was induced by the MSCs. It was reported that MSCs increased macrophage infiltration via a paracrine mechanism during wound healing after MI (*36*). There are two types of macrophages/monocytes: the classically activated proinflammatory M1 type and the alternatively activated anti-inflammatory M2 type (*37*). In our present study, many of the infiltrating macrophages were $CD163^+$ M2 macrophages (see **Figure S2b, SDC,** http://links.lww.com/TP/A849). M2 macrophages secrete several angiogenic factors promoting neovascularization (*38, 39*). Freytes et al. (*40*) reported that M2 macrophages modulated the viability of MSCs, and MSCs were reported to mediate a switch of macrophages to an anti-inflammatory activation state, which may be associated with the enhancement of cardiac function (*36, 41, 42*). Although further studies are needed, the induction of M2 macrophages may be one of the therapeutic mechanisms of MSC transplantation in MI.

The mechanisms responsible for the therapeutic effects of transplantation of allogeneic FM-MSC sheets in chronic MI are still unclear, and poor long-term survival and low differentiation rates of both types of transplanted MSC sheets are limitations of our study. In our previous study using the MI model, monolayer adipose tissue–derived MSC sheets gradually grew and developed into a thick stratum (*12*). The different results obtained in this study may have been caused by the difference in the cell sources. Several studies indicate differences between adipose tissue–derived MSCs and BM-MSCs (*43, 44*). Some studies tried to increase the therapeutic effects of cell transplantation by, for example, gene transduction or using a combination of drugs (*45–48*). Xu et al. (*49*) reported that lovastatin protected BM-MSCs from hypoxia-induced apoptosis, and Yang et al. (*50*) demonstrated that simvastatin improved the therapeutic efficacy of BM-MSC transplantation in an acute MI model by promoting cell survival and cardiovascular differentiation. These drug treatments may improve cell viability and increase the therapeutic effects of transplantation of MSC sheets in heart failure.

In conclusion, transplantation of allogeneic FM-MSC sheets improved cardiac function in a rat model of MI possibly by inducing angiogenesis and inhibiting myocardial fibrosis. The therapeutic effects were similar to those of transplanting autologous BM-MSC sheets and might be caused by the paracrine effects and the M2 macrophage induction. FM-MSC could be considered a new cell source, allowing wider clinical applications of MSC transplantation therapy. Although further experiments are needed to apply the current results to human cardiomyoplasty, transplantation of allogeneic FM-MSC sheets may provide a new therapeutic strategy for the treatment of MI.

MATERIALS AND METHODS

Animals

Male 8-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were used in this MI model. Male GFP-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan) and female GFP-transgenic Sprague–Dawley rats (Japan SLC) were also used for the harvest of transplanted cells. The experimental protocols were approved by the Animal Care Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan).

Expansion of FM-MSCs and BM-MSCs

The isolation and expansion of FM-MSCs and BM-MSCs were performed as described previously (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849) (*8*). In all experiments, FM-MSCs and BM-MSCs were used at passages 4 to 8.

Preparation of MSC Sheets

To prepare MSC sheets, we used 35-mm temperature-responsive dish (UpCell, CellSeed, Tokyo, Japan). Preparation of MSC sheets was performed as described previously (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849) (*12*).

Model of MI

To create an MI model, male Lewis rats (220–250 g) were anesthetized, and the left coronary artery was ligated, as described previously (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849) (*12*). The sham group underwent thoracotomy and cardiac exposure without coronary ligation.

We randomly assigned the rats to four groups: (a) rats with chronic heart failure that underwent transplantation with allogeneic FM-MSC sheets (allo-FM-MSC–transplanted MI group; n=10), (b) rats with chronic heart failure that underwent transplantation with autologous BM-MSC sheets (auto-BM-MSC–transplanted MI group; n=10), (c) rats with chronic heart failure without transplantation (untreated MI group; n=10), and (d) sham-operated rats without transplantation (sham group; n=10). Four weeks after coronary ligation, the allo-FM-MSC–transplanted MI group and auto-BM-MSC–transplanted MI group underwent transplantation with the respective two-layered cell sheets. The sheets were placed on the anterior wall, including the scar area, and then covered with oxidized regenerated cellulose (INTERCEED [TC7], Johnson & Johnson Medical, Tokyo, Japan). The other two groups underwent the same operative procedures without transplantation.

Hemodynamic Studies

Hemodynamic studies were performed 8 weeks after coronary ligation (4 weeks after transplantation (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849).

Echocardiographic Studies

Echocardiography was performed 4 weeks (before transplantation) and 8 weeks (4 weeks after transplantation) after coronary ligation (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849).

Immunohistochemical Studies

Immunohistochemical details (see **Materials and Methods, SDC**, http://links.lww.com/TP/A849).

Statistical Analysis

Data are expressed as mean±SE. Analysis of variance was used to compare each variable between groups, and the post hoc Tukey's test was used to locate significant differences. Differences were considered significant at $P<0.05$.

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Extracellular High Mobility Group Box 1 Plays a Role in the Effect of Bone Marrow Mononuclear Cell Transplantation for Heart Failure

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Abstract

Transplantation of unfractionated bone marrow mononuclear cells (BMCs) repairs and/or regenerates the damaged myocardium allegedly due to secretion from surviving BMCs (paracrine effect). However, donor cell survival after transplantation is known to be markedly poor. This discrepancy led us to hypothesize that dead donor BMCs might also contribute to the therapeutic benefits from BMC transplantation. High mobility group box 1 (HMGB1) is a nuclear protein that stabilizes nucleosomes, and also acts as a multi-functional cytokine when released from damaged cells. We thus studied the role of extracellular HMGB1 in the effect of BMC transplantation for heart failure. Four weeks after coronary artery ligation in female rats, syngeneic male BMCs (or PBS only as control) were intramyocardially injected with/without anti-HMGB1 antibody or control IgG. One hour after injection, ELISA showed that circulating extracellular HMGB1 levels were elevated after BMC transplantation compared to the PBS injection. Quantitative donor cell survival assessed by PCR for male-specific *sry* gene at days 3 and 28 was similarly poor. Echocardiography and catheterization showed enhanced cardiac function after BMC transplantation compared to PBS injection at day 28, while this effect was abolished by antibody-neutralization of HMGB1. BMC transplantation reduced post-infarction fibrosis, improved neovascularization, and increased proliferation, while all these effects in repairing the failing myocardium were eliminated by HMGB1-inhibition. Furthermore, BMC transplantation drove the macrophage polarization towards alternatively-activated, anti-inflammatory M2 macrophages in the heart at day 3, while this was abolished by HMGB1-inhibition. Quantitative RT-PCR showed that BMC transplantation upregulated expression of an anti-inflammatory cytokine *IL-10* in the heart at day 3 compared to PBS injection. In contrast, neutralizing HMGB1 by antibody-treatment suppressed this anti-inflammatory expression. These data suggest that extracellular HMGB1 contributes to the effect of BMC transplantation to recover the damaged myocardium by favorably modulating innate immunity in heart failure.

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Introduction

Transplantation of stem or progenitor cells is an emerging approach to repair and/or regenerate damaged myocardium undergoing adverse ventricular remodeling. Unfractionated bone marrow mononuclear cells (BMCs) contain several kinds of stem/progenitor cells and are the most frequently used donor cell type in clinical cell therapy to the heart [1]. The therapeutic effect of BMC transplantation in not only acute myocardial infarction (MI) but also post-MI chronic heart failure (ischemic cardiomyopathy) has been confirmed in animal and human studies [1–3]. Because injected BMCs do not vigorously differentiate to functioning cardiomyocytes or vascular cells *in vivo*, the major mechanism of the therapeutic effects is proposed to be their secretion of cytokines, chemokines and growth factors that help repair of the damaged myocardium suffering post-MI adverse remodeling [1–3]. However, the precise mechanism of this "paracrine effect" remains uncertain.

Interestingly, cardiac function recovery by BMC transplantation occurs despite of markedly poor donor cell survival [1,3,4]. It has also been shown that active secretion from BMCs is less extensive compared to other donor cell types [5,6]. It was also reported that injection of extract of dead BMCs by freeze-thaw cycles induces the similar therapeutic effect to injection of living BMCs [7]. These findings led us to hypothesize that dead donor BMCs might be a supplementary or alternative source of the paracrine mediators, which could contribute to the repair of the failing myocardium.

High-mobility group box 1 (HMGB1) was initially identified as a nuclear protein that regulates transcriptional factors to stabilize the nucleosome [8]. This molecule is also known to be actively secreted from activated inflammatory cells and also passively

released from dead cells [9–11]. Extracellular HMGB1 induces and intensifies inflammation in most cases, while it can also operate to attenuate inflammation and enhance the healing of damaged tissues, according to the form/amount of HMGB1 and nature of the tissues [9–12]. In the heart, there is increasing evidence that extracellular HMGB1 attenuates myocardial damage and induces recovery/regeneration [13–18], though there are contradicting reports [19,20]. We have demonstrated that HMGB1 administration achieved the similar benefits to the BMC-mediated paracrine effects, including decreased fibrosis, increased vascular formation, attenuated cardiomyocyte hypertrophy, and attenuated inflammation in a rat ischemic cardiomyopathy model [17]. It has also been reported that extracellular HMGB1 augments tissue regeneration through activating endogenous progenitor cells [15,21].

Collectively, these data formed a hypothesis that extracellular HMGB1 released from dead donor cells contributes to the paracrine effect of BMC transplantation to repair the post-MI failing myocardium and to improve cardiac performance.

Materials and Methods

Ethics Statement

All studies were performed with the approval of the UK Home Office (Project Licence Number: 70/7254). The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US NIH Publication, 1996). All animal surgery was performed under inhalation anesthesia of isoflurane and administration of buprenorphine hydrochloride was made just after surgery to reduce postoperative pain, and all efforts were made to minimize suffering. Surgical procedures, cardiac function measurement, and sample analyses were performed in a blinded manner.

BMC Collection

Bone marrow was isolated from both femurs and tibias of male Lewis rats (150–200 g; Charles River, UK), from which BMCs (mononuclear cells) were purified by Ficoll-Paque gradient centrifugation (GE Healthcare) as previously described [3]. Flow cytometry analysis (FACSAria, BD Biosciences) using monoclonal anti-rat CD34 (Santa-Cruz) and anti-rat CD45 (BD Pharmingen) antibodies showed that 4.6±1.7% of the BMCs were positive for CD34 and 75.5±4.3% were positive for CD45 (**Figure S1**). To trace the injected cells, BMCs were labeled with CM-DiI (Molecular Probes) before transplantation according to the company's protocol. The viability of donor BMCs just before injection measured by trypan blue staining was 97.1±0.6% (n = 11 animals).

Assessment of Cardiac Function

Cardiac function and dimensions pre and post treatment were measured by using echocardiography (Vevo−770, VisualSonics) as previously described [3,17]. Diastolic and systolic LV endocardial areas at the papillary muscle level were measured from parasternal short-axis views, from which LV fractional area change (LVFAC) was calculated. Post treatment hemodynamics parameters were measured by catheterization (SRP−320/PVAN3.2, Millar Instruments and Chart 5 software, ADInstruments) as described previously [22].

Generation of Ischemic Cardiomyopathy and Cell Transplantation in Rat

Female Lewis rats (150–200 g, Charles River) underwent left coronary artery ligation as described previously [3,17,23,24]. Four weeks later, the animals that showed appropriate cardiac dysfunction (LVFAC 22–32%; base line in intact rats = 61.6±1.7% [n = 5]) by echocardiography were chosen and randomly assigned to 4 treatment groups; intramyocardial injection of 1×10^7 syngeneic male BMCs (BMC group), injection of BMCs with 50 μg anti-HMGB1 neutralizing antibody (Medical & Biological Laboratories; AB group), injection of BMCs with 50 μg control IgG (Sigma-Aldrich; IgG group), and injection of PBS only (CON group). BMCs were suspended in 200 μl of PBS and intramyocardial injection was performed into 2 sites (100 μl each) of the LV free wall, targeting the border areas [3].

To optimize the antibody dose, 1×10^7 BMCs were injected with 0, 10, 50, or 100 μg of anti-HMGB1 antibody in the same model ($n\geq3$). At day 28, LVFAC was 31.5±1.2, 30.3±1.5, 26.6±1.3, and 26.4±1.8%, respectively. Then, 50 μg antibody was used in the main study.

Detection of Released HMGB1

Peripheral blood was collected, from which serum was obtained by centrifugation. HMGB1 levels in the serum were determined in duplicate using a commercial ELISA kit (IBL international GMBH) according to the manufacture's instruction.

Histological Analysis

The hearts were excised, fixed with 4% paraformaldehyde, embedded in OCT compound, and quickly frozen in liquid nitrogen. Cryosections were cut and incubated with biotin conjugated Griffonia simplicifolia lectin I-isolectin B4 (1:100, Vector), monoclonal anti-rat CD68 antibody (1:100, AbD Serotec), monoclonal anti-rat CD86 antibody (1:50, BD), monoclonal anti-rat CD163 antibody (1:100, AbD Serotec), monoclonal anti-rat Ki-67 antibody (1:50, DakoCytomation), and/or polyclonal anti-rat cardiac troponin-T (cTnT) antibody (1:200, HyTest) followed by visualization using appropriate fluorophore-conjugated secondary antibodies (Molecular Probes). Samples were observed by a fluorescence microscopy (BZ8000, Keyence) with or without nuclear counter-staining using 4′, 6-diamidino-2-phenylindole (DAPI). Ten different fields were randomly selected in each border area of the samples and assessed. Another set of sections were stained with 0.1% picrosirius red, which enabled calculation of extracellular collagen volume fraction in border area by using NIH image-analysis software [3,17,22].

Quantitative Analysis of Donor Cell Survival

Genomic DNA was extracted from the whole LV samples of female rats. To detect donor cell (male) survival, expression of the Y chromosome-specific *sry* gene in these samples was assessed by real-time polymerase chain reaction (PCR; Prism 7900HT, Applied Biosystems). The *sry* levels were normalized to the DNA amount using the autosomal single copy gene, oesteopontin. The number of surviving donor cells was estimated by correcting the relative *sry* expression using a standard curve as previously described [3,17,22].

Measurements of Myocardial Gene Expression

Total RNA was extracted from the whole LV samples and assessed for myocardial expression of *IL-1β*, *TNF-α*, and *IL-10* by quantitative RT-PCR (Prism 7900HT, Applied Biosystems) as previously described [22]. TaqMan primers and probes were

purchased from Applied Biosystems. Expression was normalized using *Ubiquitin C*.

Statistical Analysis

All values are expressed as mean±SEM. Statistical comparison of the data was performed using the student's unpaired *t*-test for the analysis of circulating HMGB1 levels. All other data were analyzed with one-way ANOVA followed by Fisher's post-hoc analysis to compare groups. A value of $p<0.05$ was considered statistically significant.

Results

Poor Donor Cell Survival and Increased Extracellular HMGB1 After BMC Transplantation

Female rats suffering ischemic cardiomyopathy were randomly assigned to 4 groups; intramyocardial injection of syngeneic male BMCs (BMC group), intramyocardial injection of male BMCs with anti-HMGB1 neutralizing antibody (AB group), intramyocardial injection of male BMCs with control IgG (IgG group), and intramyocardial injection of PBS only (CON group). After each treatment, quantitative PCR for the male-specific *sry* gene demonstrated that donor cell survival after BMC transplantation was poor similarly in the BMC, AB, and IgG groups; below 10% at day 3, further decreasing to below 1% by day 28 (**Figure 1A**). Histological analysis detected islet-like clusters of donor cells at day 3 after BMC transplantation (**Figure 1B**). ELISA showed that the circulating extracellular HMGB1 level was 2.5-fold elevated one hour after BMC transplantation, compared to the PBS injection control (**Figure 1C**).

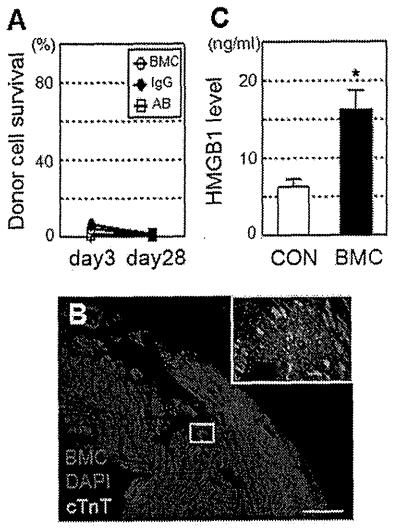

Figure 1. Poor donor cell survival and HMGB1 leakage after BMC transplantation. (**A**) Quantitative PCR for the male specific *sry* gene showed that the survival of male donor cells in female hearts was poor similarly in the BMC (BMC injection), IgG (BMC+control IgG injection), and AB (BMC+anti-HMGB1 antibody injection) groups at both days 3 and 28; n=5~7 in each point. (**B**) Clusters of DiI-labeled (red) donor BMCs were detected in the heart at day 3 after BMC transplantation. A higher magnification image of the yellow frame is shown. Green=cardiomyocytes (cTnT); blue=nuclei (DAPI). Scale bar=300 μm. (**C**) ELISA showed that the circulating HMGB1 level was increased at 1 hour in the BMC group compared to the PBS injection control (CON group). *:$p<0.05$ *versus* the CON group, mean±SEM for n=5 each.
doi:10.1371/journal.pone.0076908.g001

Abolished BMC Transplantation-induced Cardiac Function Recovery by HMGB1-inhibition

Four weeks after BMC transplantation (BMC group), echocardiography and cardiac catheterization consistently demonstrated that both systolic and diastolic LV function, in terms of LV fractional area change, max and min dP/dt, and systolic pressure, was improved compared to the control (CON group; **Figure 2**). Enlargement of LV systolic endocardial area in the control group was attenuated by BMC transplantation. Of note, these effects were largely abolished by HMGB1-inhibition (AB group), but not by control IgG administration (IgG group), indicating an important role of extracellular HMGB1 in the therapeutic benefits of BMC transplantation.

Elimination of BMC Transplantation-induced Tissue Recovery by HMGB1-inhibition

To investigate the mechanism by which extracellular HMGB1 released in BMC transplantation improved post-MI cardiac function, we performed a set of histological studies with a focus on the paracrine effect. Consistent to previous reports [3], BMC transplantation (BMC group) attenuated post-MI pathological fibrosis, improved neovascular formation, and increased proliferation activity in the border areas at day 28, compared to the control (CON group; **Figure 3 and Figure S2**). All these paracrine effects were, however, eliminated by HMGB1-inhibition (AB group), but not by IgG administration (IgG group), corresponding to the cardiac function change as shown in Figure 2.

Modulation of M2/M1 Macrophage Polarization by BMC Transplantation through HMGB1

Additional immunolabeling showed that BMC transplantation increased myocardial accumulation of $CD68^+$ pan-macrophages compared to the control (**Figure 4A and Figure S3A, B**). Here, the increase in $CD86^+$ classically-activated pro-inflammatory (M1) macrophages was trivial (**Figure 4B and Figure S3D, E**), while the enhancement of $CD163^+$ alternatively-activated (M2) macrophages was more obvious (**Figure 4C and Figure S3G, H**). As a result, the ratio of M2/M1 macrophage in the BMC group (92.9/36.9=2.52) was increased from 56.3/28.4=1.98 in the CON group. Of note, HMGB1-inhibition abolished the enhancement of $CD163^+$ M2 macrophages (**Figure 4C and Figure S3I**) and exacerbated the increase in $CD86^+$ M1 macrophages (**Figure 4B and Figure S3F**), thus largely reducing the M2/M1 ratio to 67.6/62.9=1.07. These results suggest that the HMGB1-mediated shift of macrophage polarization towards anti-inflammatory M2 macrophages might play a role in the BMC transplantation-induced myocardial recovery.

Quantitative RT-PCR showed that myocardial expression of the anti-inflammatory cytokine, *IL-10*, tended to be elevated by BMC transplantation compared to the control, while this was totally eliminated by inhibiting HMGB1 (**Figure 4D**). IL-10 is known to be secreted by alternatively activated M2 macrophages and also by Th2 cells that induce M2 macrophage differentiation [9,25,26]. The expression of *IL-1β* or *TNF-α* was not affected by either BMC transplantation or HMGB1-inhibition (**Figure 4E, F**).

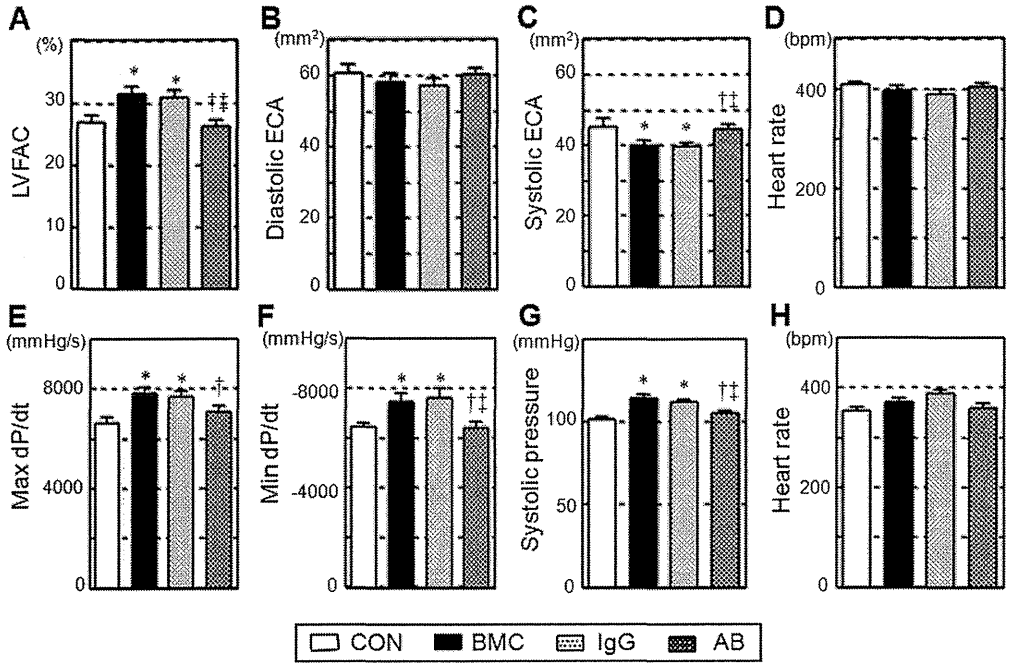

Figure 2. Abolished BMC transplantation-induced cardiac function recovery by HMGB1-inhibition. Cardiac parameters were measured by echocardiography (**A–D**) and catheterization (**E–H**) on day 28 after each treatment. Cardiac function was improved by BMC transplantation (BMC group) compared to the PBS injection control (CON group), while this effect was eliminated by antibody neutralization of HMGB1 (AB group), but not by control IgG administration (IgG group). LVFAC, left ventricular fractional area change; ECA, endocardial area. *:$p<0.05$ *versus* the CON group, †:$p<0.05$ *versus* the BMC group. ‡:$p<0.05$ *versus* the IgG group, mean±SEM for n = 8~10 in each group.
doi:10.1371/journal.pone.0076908.g002

Discussion

Using a post-MI ischemic cardiomyopathy model in rat, we demonstrated that the BMC transplantation-mediated benefits, including increased neovascular formation, reduced collagen deposition, increased proliferation activity, favorable modulation of macrophage polarization, and resultant improvement of cardiac function, were all eliminated by antibody-neutralization of HMGB1. These data suggest that extracellular HMGB1 plays a role in the effects of BMC transplantation to recover the failing myocardium undergoing post-MI adverse remodeling and to improve global cardiac function. This finding is validated by the consistency with the previous report demonstrating that administration of recombinant HMGB1 protein achieved the same benefits as the BMC transplantation-mediated effects using the similar ischemic heart failure model in rats [17].

In view of the origin of extracellular HMGB1 occurring after BMC transplantation into post MI chronic heart failure, there are 4 theoretically possible sources: (i) passive release from dead donor BMCs, (ii) passive release from dead host (endogenous) cells, (iii) active secretion from surviving donor BMCs, and (iv) active secretion from host (endogenous) cells. It is likely that the major origin may be (i) passive release from dead donor BMCs, given the following information. [I] Survival of donor BMCs was largely limited, indicating that there was a considerable amount of donor cell death; [II] In this model of ischemic cardiomyopathy, death/damage of host cells in the heart and other organs by BMC injection is unlikely to be substantial; [III] There was an increased level of circulating HMGB1 as early as 1 hour after BMC injection. This time course is too rapid for inflammatory cells to actively secrete HMGB1 *via* transcription after stimulation [9,27]. Having said these, we could not eliminate the possibility that extracellular HMGB1 from other sources, particularly from endogenous sources (host cells), might contribute to the paracrine effect of BMC transplantation. Experiments using HMGB1-deficient BMCs, either by knockout or knockdown, as donor would provide useful information to this point. However, HMGB1 knockout mice die immediately after birth [28], while on the other hand reproducible and satisfactory knockdown in primary rat unfractionated BMCs has not been established.

The role of extracellular HMGB1 to attenuate myocardial damage and to induce recovery and/or regeneration remains controversial [13–20]. This discrepancy may be relevant to different types of HMGB1 and different conditions of the host myocardium. In the settings of acute MI (without cell transplantation), a large amount of HMGB1 is actively secreted from accumulated inflammatory cells in addition to HMGB1 passively released from a large number of dead host cardiac cells. The dynamics and functions of these different types of HMGB1 are likely to be distinct, due to different phosphorylation, acetylation, and formation of complexes by binding other pathogenic molecules [9,11]. Delicate balance between these types of extracellular HMGB1 may affect the overall effect, whether beneficial or harmful, of HMGB1. Our study therefore used a post-MI ischemic cardiomyopathy model to exclude these contaminating factors. The degree of HMGB1 both from inflammatory cells and dead host cardiac cells in this model is presumed to be much less than that in acute MI settings, and the frequency of host cardiac cell death by BMC injection is also negligible, compared to donor cell death.

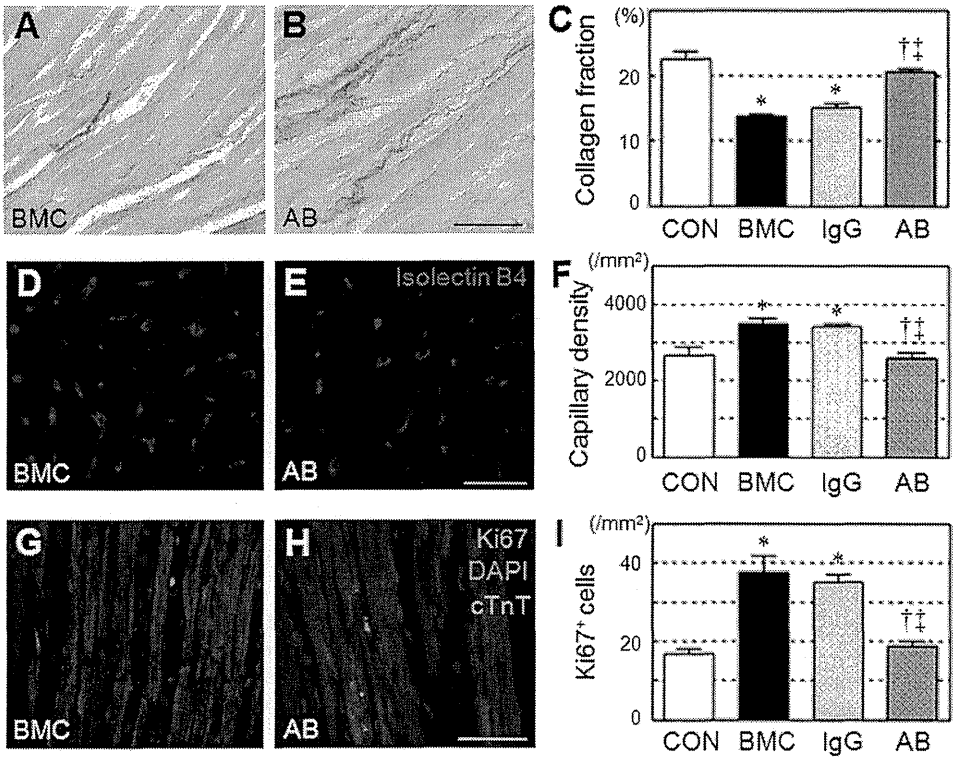

Figure 3. Eliminated BMC transplantation-induced tissue recovery by HMGB1-inhibition. Reduced extracellular collagen deposition (**A–C;** picrosirius red = red), increased capillary density (**D–F;** Isolectin B4 = red), and increased proliferation (**G–I;** Ki67 = red; nuclei = blue; cTnT = green) were observed in the border areas at day 28 after BMC transplantation (BMC group), compared to the PBS control (CON group). These effects were all abolished by anti-HMGB1 antibody neutralization (AB group), but not by control IgG administration (IgG group). Representative images of only BMC and AB groups are present (see **Figure S2** for additional images). Scale bars = 50 μm in **A, B, G, H** and 30 μm in **D, E.** *:$p<0.05$ *versus* the CON group, †:$p<0.05$ *versus* the BMC group, ‡:$p<0.05$ *versus* the IgG group, mean±SEM for n = 5~7 in each group.
doi:10.1371/journal.pone.0076908.g003

It will be interesting to investigate whether the present results in unfractionated BMCs are applicable to other types of donor cells. There are published data in other cell types that appear to be contradicting to our findings in BMCs at a glance. Ziebart *et al.* have reported that the persistence of donor cells contributes to the therapeutic effect of transplantation of endothelial progenitor cells

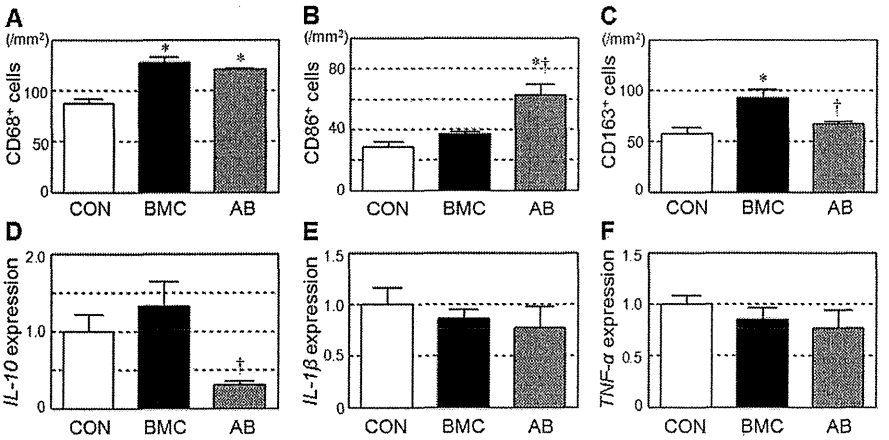

Figure 4. Modulation of innate immunity by BMC transplantation via released HMGB1. Accumulation of $CD68^+$ pan-macrophages (**A**), $CD86^+$ classically-activated pro-inflammatory M1 macrophages (**B**), and $CD163^+$ alternatively-activated anti-inflammatory M2 macrophages (**C**) in the border areas at day 3 after each treatment was assessed by immunolabeling. See **Figure S3** for representative images. Myocardial expression of *IL-10* (**D**), *IL-1β* (**E**)), and *TNF-α* (**F**) at day 3 after each treatment was measured by quantitative RT-PCR. *:$p<0.05$ *versus* the CON group, †:$p<0.05$ *versus* the BMC group, mean±SEM for n = 5~7 in each group.
doi:10.1371/journal.pone.0076908.g004

by using the inducible suicide gene [29]. Laflamme *et al.* have demonstrated that increase of donor cell presence (thus reducing donor cell death) by the treatment with pro-survival factors enhances therapeutic effects of transplantation of embryonic stem cell-derived cardiomyocytes [30]. However, in contrast, in the case of unfractionated BMCs, Yeghiazarians *et al.* reported that ultrasound-guided injection of extract of dead BMCs by freeze-thaw cycles achieves the similar therapeutic effect to injection of living BMCs [7], supporting our findings. This cell type-dependent controversy may suggest that the impact of HMGB1 released from dead donor cells to recover the damaged myocardium could be diluted/hidden in the case of transplantation of "more proficient" cells such as endothelial progenitor cells and embryonic stem cell-derived cardiomyocytes that have more substantial abilities of beneficial differentiation, secretion, and/or contraction. In addition, the extent and/or types of donor cell death after transplantation may vary according to donor cell types, affecting the release of HMGB1. Differences in the condition of the host heart, *i.e.* acute MI *versus* post-MI ischemic cardiomyopathy, may also influence the impact of extracellular HMGB1 from donor cells. Further studies to elucidate the role of extracellular HMGB1 in different donor cell types in the same experimental setting are warranted.

Macrophages are an important player in the progress and recovery of post-MI adverse ventricular remodeling [31,32]. It has also been shown that HMGB1 and receptor for advanced glycation endproducts signaling play a role in the macrophage-involved tissue repair mechanism in the peripheral nerve [33]. Recent research has shown that macrophages can be functionally polarized into classically-activated M1 (pro-inflammatory) or alternatively-activated M2 (anti-inflammation and tissue healing) phenotypes according to the environmental condition [9,26]. Stimulation with IFN-γ or TNF-α drives the macrophages into the M1 phenotype, which is characterized by a strong pro-inflammatory ability. In contrast, exposure to IL-4 or IL-13 generates M2 macrophages, which attenuate inflammation and enhance tissue recovery and healing. Importance of this polarization balance in the repair of damaged organs, including the heart, has been reported [9,26]. Our results here uncovered that BMC transplantation enhanced "beneficial" M2 macrophages in the heart, for which extracellular HMGB1 was responsible. Therefore, HMGB1-mediated modulation of the macrophage's polarization towards the M2 phenotype might be a part of the mechanism by which BMC transplantation recovers the damaged myocardium and improves cardiac function. Further study should focus on elucidation of the molecular mechanism of extracellular HMGB1 to modulate the M1/M2 macrophage polarization, in a simpler model.

A limitation of this study may be that our conclusion is based on the antibody neutralization experiments, which might carry a risk (though unlikely) of unexpected artifacts, such as unpredictable actions of the immune complexes generated. Investigations using HMGB1-deficient cells, either by knockout or knockdown, could add useful validation of our results, but these were not practical due to technical limitations as discussed above. Nonetheless, the consistency with the previous evidence that administration of HMGB1 protein induces the same effects as the BMC transplantation [17] supports our results.

In summary, our results demonstrated that extracellular HMGB1, which is derived from dead donor cells at least in part, plays a role of the effect of BMC transplantation to recover the damaged tissue by favorably modulating innate immunity in heart failure. This novel proof-of-concept will imply an important clue to further understand and refine BMC transplantation therapy for heart failure.

Supporting Information

Figure S1 Characterisation of BMCs by flow cytometry analysis. Flow cytometry analysis showed that 4.6±1.7%of collected rat BMCs were positive for CD34 and 75.5±4.3% were positive for CD45. A representative image is presented.
(TIF)

Figure S2 Supplement to Figure 3; HMGB1-inhibition abolished myocardial recovery by BMC transplantation. A–D: Representative images of picrosirius red staining. Scale bar = 50 μm. **E–H:** Representative images of islectin-B4 staining (red). Scale bar = 30 μm. **I–L:** Representative images of immunofluorescent labelling with Ki-67 (red); blue = nuclei (DAPI). Scale bar = 50 μm.
(TIF)

Figure S3 Supplement to Figure 4; Inflammation was modulated by BMC transplantation through HMGB1. Representative images of immunofluorescent labelling with CD68 (**A–C**), CD86 (**D–F**), and CD163 (**G–I**). Green is for each target molecule; blue for nuclei (DAPI). Scale bars = 50 μm.
(TIF)

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Author Contributions

Conceived and designed the experiments: KS MK SRC. Performed the experiments: MK YS TN NT CI KY SF. Analyzed the data: KS MK SRC. Wrote the paper: KS MK SRC.

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