

PRE-CLINICAL RESEARCH

Macrophage Subpopulations Are Essential for Infarct Repair With and Without Stem Cell Therapy

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- Objectives** This study sought to investigate the hypothesis that the favorable effects of mesenchymal stromal cells (MSCs) on infarct repair are mediated by macrophages.
- Background** The favorable effects of MSC therapy in myocardial infarction (MI) are complex and not fully understood.
- Methods** We induced MI in mice and allocated them to bone marrow MSCs, mononuclear cells, or saline injection into the infarct, with and without early (4 h before MI) and late (3 days after MI) macrophage depletion. We then analyzed macrophage phenotype in the infarcted heart by flow cytometry and macrophage secretome in vitro. Left ventricular remodeling and global and regional function were assessed by echocardiography and speckle-tracking based strain imaging.
- Results** The MSC therapy significantly increased the percentage of reparative M2 macrophages (F4/80⁺CD206⁺) in the infarcted myocardium, compared with mononuclear- and saline-treated hearts, 3 and 4 days after MI. Macrophage cytokine secretion, relevant to infarct healing and repair, was significantly increased after MSC therapy, or incubation with MSCs or MSC supernatant. Significantly, with and without MSC therapy, transient macrophage depletion increased mortality 30 days after MI. Furthermore, early macrophage depletion produced the greatest negative effect on infarct size and left ventricular remodeling and function, as well as a significant incidence of left ventricular thrombus formation. These deleterious effects were attenuated with macrophage restoration and MSC therapy.
- Conclusions** Some of the protective effects of MSCs on infarct repair are mediated by macrophages, which are essential for early healing and repair. Thus, targeting macrophages could be a novel strategy to improve infarct healing and repair. (J Am Coll Cardiol 2013;62:1890–901) © 2013 by the American College of Cardiology Foundation

One of the major challenges in the management of acute myocardial infarction (AMI) is to improve the healing process, particularly in elderly and sick persons whose repair ability

is impaired (1,2). Mesenchymal stromal cells (MSCs) have been proposed to improve infarct repair (3), but their mechanism of action is complex and not fully understood (4,5).

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One cell type with which MSCs might interact during infarct healing is the macrophage (6). Although widely recognized as contributors to the pathogenesis of atherosclerosis, plaque build-up, and AMI, monocytes and macrophages also contribute to angiogenesis, infarct healing, and tissue remodeling through their secretion of anti-inflammatory and angiogenic cytokines (7–9). In previous studies, we found macrophage collections at the sites of cell transplantation in the infarct (4,10). Interestingly, despite very early and massive loss of the transplanted cells, the favorable effects of cells on left ventricular (LV) remodeling and function were preserved (4,10). These unique phenomena motivated us to investigate

the interaction between MSCs and macrophages during infarct repair.

In response to activating signals, macrophages are “re-educated” and become polarized into either a M1 phenotype (proinflammatory) or M2 phenotype (anti-inflammatory). The M1 macrophages are characterized by production of proinflammatory cytokines including tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , whereas M2 macrophages are commonly associated with secretion of IL-10 (11–13). MSCs can modify macrophages from an M1 to an M2 phenotype *in vitro* (14–17) and *in vivo* (18). However, it is unclear whether macrophage modulation by MSCs can affect infarct repair. Based on our work and that of others, we hypothesized that the interaction between transplanted MSCs and infarct macrophages may play a role in infarct repair, and could be exploited to improve LV remodeling and function. The aim of the present study was to test this hypothesis in a mouse model of MI.

Methods

Detailed descriptions of material and methods are presented in the [Online Appendix](#). The MSCs were isolated from bone marrow (BM) of female Balb/C mice (age 12 weeks, weight 20 g; Harlan, Jerusalem, Israel). To characterize the BM MSCs, we harvested MSCs from the fifth passage and analyzed the cells by Calibur flow cytometer (BD Biosciences, San Jose, California). The MSCs of the fifth passage exhibited typical surface markers such as CD105 (44%) and Sca-1 (82%), but not markers of hemopoietic cells such as CD45, CD31, TER-119, and F4/80. Furthermore, these cells differentiated into adipocytes, osteoblasts, and chondrocytes *in vitro* ([Online Fig. 1](#)).

To determine the role of macrophage subsets in infarct repair and MSC therapy, female Balb/C mice were subjected to MI by left main coronary artery occlusion ([Online Table 1](#)). Then, we induced macrophage depletion by intravenous injection of 0.1 ml clodronate liposomes (CL), either 4 h before MI (early depletion) or on the third day after MI (late depletion). Normal mice ($n = 5$) received intravenous injections of CL using the same injection protocol to exclude the possibility of a toxic effect of CL. One minute after artery occlusion, the ischemic area was identified, and either MSCs (1×10^5), mononuclear cells (MNCs [1×10^5]), or saline (20 μ l) were injected into the border zone. Conventional echocardiography (1, 7, and 30 days after MI) and speckle-tracking based strain imaging (1 and 7 days after MI) were performed with a special echocardiography system (Vevo 2100 Imaging System, VisualSonics, Toronto, Ontario, Canada), equipped with a 30 MHz phased transducer.

To determine macrophage subsets and the effect of MSCs on macrophage polarization and function after MI, we used flow cytometry, enzyme-linked immunosorbent assay, and RayBio mouse cytokine antibody array G series (RayBiotech, Norcross, Georgia) exploring 62 different cytokine profiles. To determine culture homogeneity, cells

were stained with anti-mouse F4/80 or scrubbed and stained with APC-conjugated anti-mouse F4/80 and analyzed by flow cytometry.

To determine the effect of MSCs on infarct size, vascularization, and inflammation, we harvested hearts 7 and 30 days after injection (after the echocardiography imaging). Hearts were sectioned and fixed, and adjacent blocks were embedded in paraffin or optimal cutting temperature compound (for cryosection) and sectioned into 5- μ m slices.

Statistical analysis. Statistical analysis was performed with the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). All variables are expressed as mean \pm SEM. Normality was tested with the Kolmogorov-Smirnov test. The Mann-Whitney U test was used (if data were not normally distributed) to compare between 2 groups. Differences between baseline and 30 days were assessed using 2-tail paired t tests. To test the hypothesis that changes in measures of LV function between 1 and 30 days varied among the experimental groups, a general linear model 2-way repeated-measures analysis of variance was used. The model included the effects of treatment, time, and treatment-by-time interaction. The Bonferroni correction was used to assess the significance of predefined comparisons at specific time points. Because of the relatively small number of animals in each group, echocardiography and speckle-tracking based strain imaging at days 1 and 7, flow cytometry, enzyme-linked immunosorbent assay, and histology data were compared by Kruskal-Wallis with Dunn’s multiple comparisons. A simple linear regression analysis was used to estimate the relationship between the number of MSCs and levels of IL-10 and TNF- α . Survival among treatment groups was compared by the log-rank (Mantel-Cox) test.

Results

MSC transplantation increased number of M2 macrophages after MI. To characterize cardiac macrophage subsets in the infarcted heart, we analyzed heart cells by flow cytometry at certain time points after MI. The number of macrophages (F4/80+ cells) in the infarcted heart increased progressively and peaked at day 4 after MI ([Fig. 1A](#)). Notably, MSC transplantation reduced the percentage of infarct macrophages ([Fig. 1A](#)).

Two macrophage subsets were identified in the infarcted hearts: M1 macrophages characterized by double staining for F4/80 and CD86, and M2 macrophages characterized by double staining for F4/80 and CD206. Without intervention, M1 macrophages were the dominant population (>50%) on day 1 after MI, and their percentage remained

Abbreviations and Acronyms

AMI	= acute myocardial infarction
BM	= bone marrow
CL	= clodronate liposomes
IL	= interleukin
LV	= left ventricle
MNC	= mononuclear cell
MSC	= mesenchymal stromal cells
TNF	= tumor necrosis factor

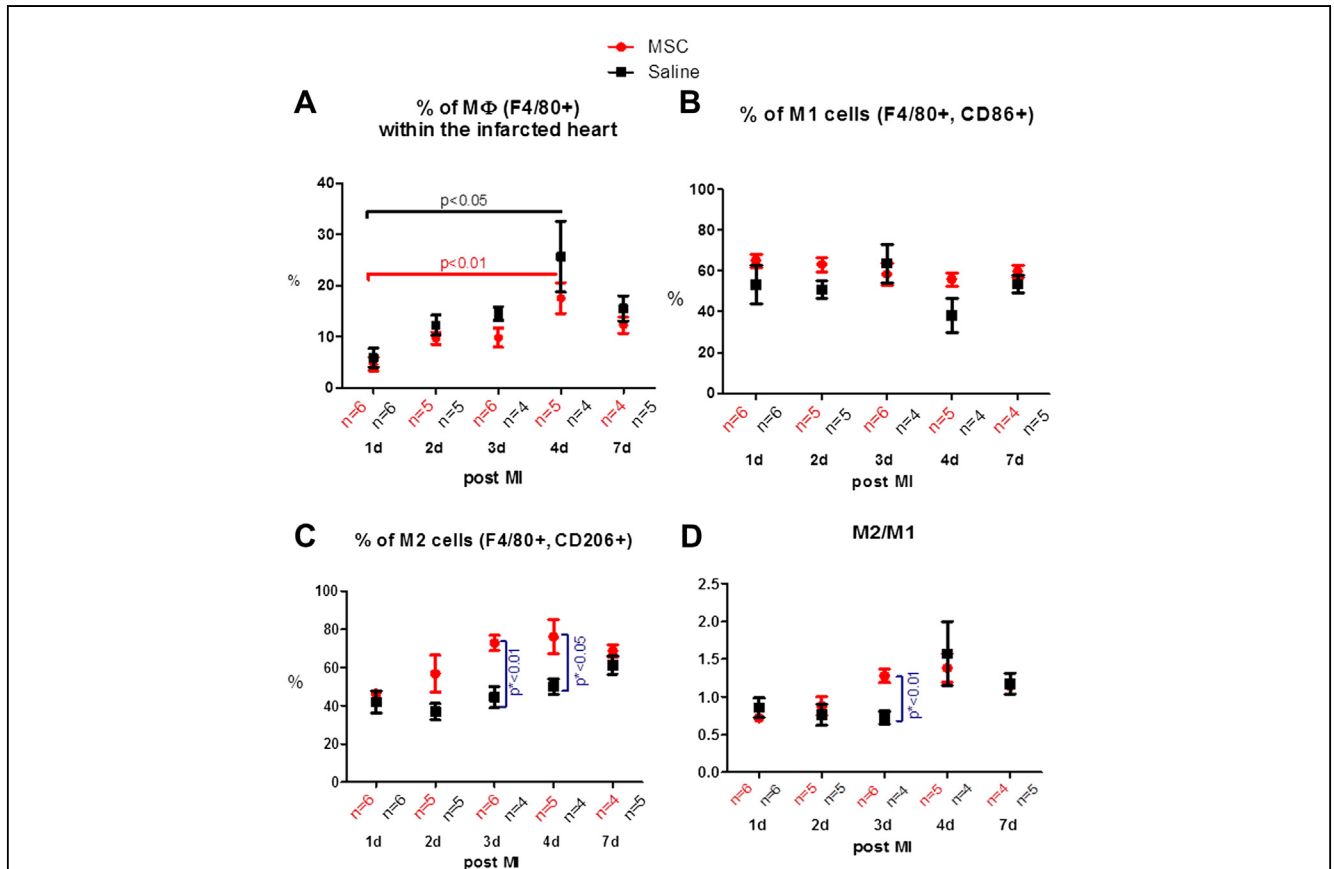


Figure 1 MSCs Increased Percentage of M2 Macrophages in the Infarcted Heart

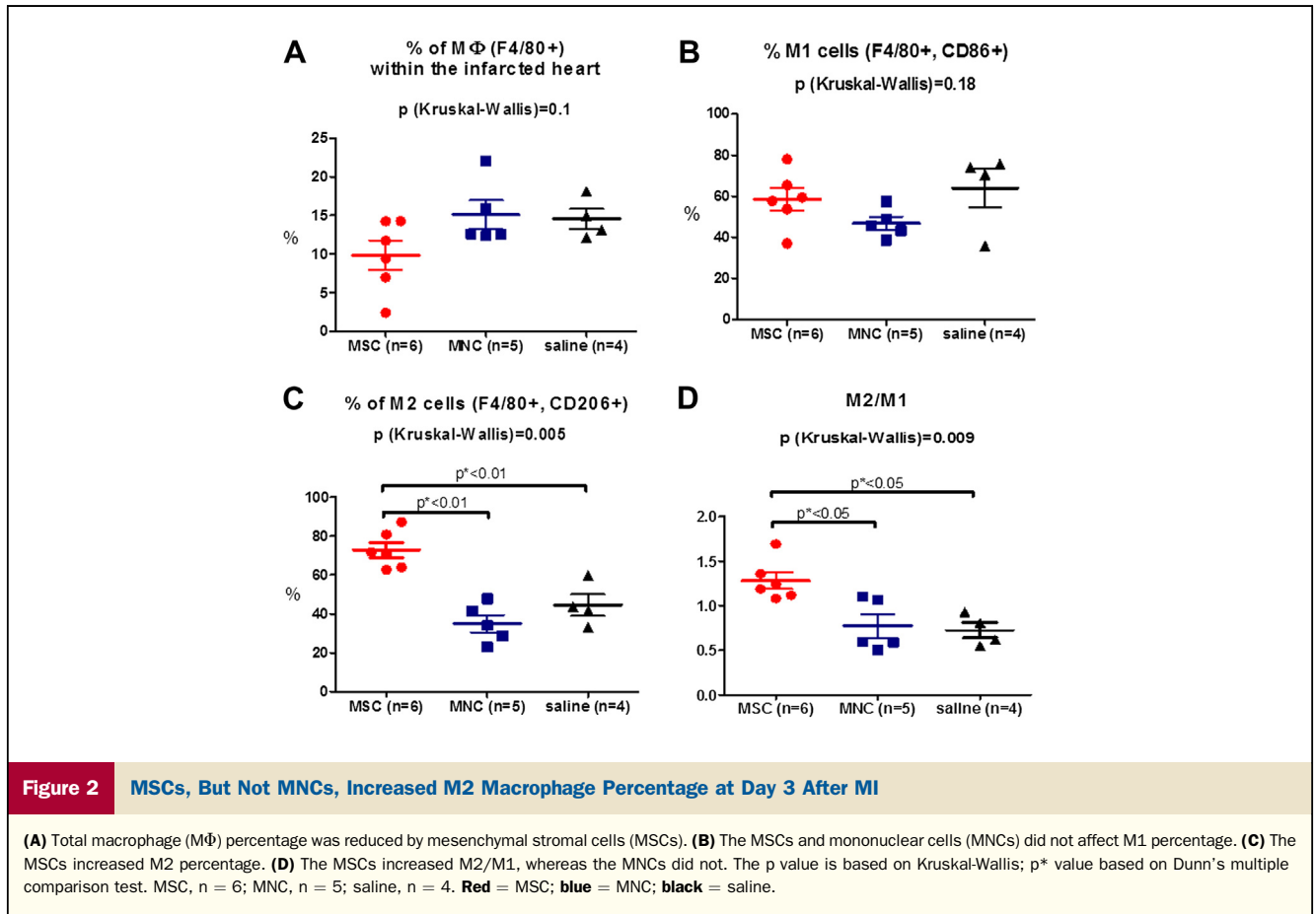
(A) There was a significant increase in macrophage (MΦ) accumulation in the infarcted heart in the first 4 days after myocardial infarction (MI). However, mesenchymal stromal cells (MSCs) reduced the number of macrophages in the infarcted heart. (B) The M1 percentage was not affected by MSCs. (C) The MSCs increased the M2 percentage. (D) The MSCs increased the M2/M1 ratio 3 days after MI. The p value is based on Dunn's multiple comparison test; p* value based on Mann-Whitney test; n = 4 to 6. Red = MSC; black = saline.

relatively constant during the first week after MI (Fig. 1B). By contrast, the percentage of M2 macrophages in the infarcted heart increased on day 4 ($47 \pm 4\%$) and even more so on day 7 ($61 \pm 4\%$) after MI ($p = 0.04$) (Fig. 1C). Significantly, MSC therapy increased the percentage of M2 macrophages by 63% and 52%, at 3 and 4 days after MI, compared with control (Fig. 1C) ($p < 0.01$; $p < 0.05$). Moreover, 3 days after MI, MSC transplantation increased the M2/M1 ratio by 77%, compared with saline ($p < 0.01$) (Fig. 1D). Interestingly, the percent of M1 plus M2 was sometimes $>100\%$, suggesting that during transition from M1 to M2, some “intermediate” macrophages expressed both M1 and M2 markers.

We then evaluated cytokine secretion from infarct macrophages with and without MSC therapy. Macrophages were isolated from the hearts, 3 days after MI and seeded on a culture dish. After enrichment, we obtained 80% macrophages in culture (Online Figs. 2a and 2b). After 1 day in culture, MSC-treated macrophages increased secretion of anti-inflammatory cytokine IL-10 by 1.94-fold (Online Fig. 2c.i), and Th2-related cytokines IL-5 and GM-CSF

by 2.6-fold and 1.84-fold, compared with saline-treated macrophages (Online Fig. 2c.ii and 2c.iii). Conversely, in this experiment, TNF- α secretion was not affected by MSCs (Online Fig. 2c.iv).

We next sought to determine whether the effect of MSCs on infarct macrophage polarization was specific or whether it could also be elicited by BM MNCs. We found that although MSCs reduced the overall percentage of macrophages (F4/80+ cells) in the infarcted heart by 33%, at day 3 after MI ($9.8 \pm 1.8\%$ vs. $14.5 \pm 1.3\%$, $p = 0.08$) (Fig. 2A), they significantly increased the percentage of M2 cells by 63% compared with saline ($p < 0.01$) (Fig. 2C). By contrast, MNCs reduced the percentage of M2 macrophages by 22% (Fig. 2C). The percentage of M1 macrophages in the infarcted heart was not affected by MSCs or MNCs (Fig. 2B). Subsequently, MSC transplantation increased the M2/M1 ratio, by 66% and 77% compared with MNC transplantation and saline injection ($p < 0.05$) (Fig. 2D). Thus, MSCs, but not MNCs, switch infarct macrophages into a reparative (M2) phenotype.



MSCs modulated macrophage secretome in vitro. To assess the interaction between macrophages and MSCs, we co-cultured BM MSCs and peritoneal macrophages for 3 days. We found that MSCs stimulate IL-10 and TNF- α secretion in a dose-dependent manner after 3 days (Online Fig. 3). We then sought to determine the source of these cytokines. We carried out an antibody-based protein array analysis on 5 different culture groups (Online Table 2). Co-cultures either with MSCs or MSC supernatant amplified macrophage secretion of M2-related, reparative, and angiogenic cytokines such as platelet factor 4 (involved in wound healing) by 144% and 72%, and vascular endothelial growth factor by 40%, compared with the sum of cytokine secreted from macrophages plus MSCs. The highest levels of reparative cytokines were obtained from a mixed culture of macrophages and MSCs, suggesting that the interaction was amplified by direct cell-to-cell contact. Interestingly, the interaction between macrophages and MSCs also stimulated secretion of IL-6 by 334% (Online Table 2). This cytokine is typical for both M1 and M2b macrophages and also relevant to tissue response to injury, healing and repair. Additionally, MSCs increase or decrease the secretion of cytokines that affect the immune system, particularly lymphocyte activation (Online Table 2).

Macrophage depletion attenuated the favorable effects of MSC therapy. Significantly, post-MI mortality after 30 days was highest after transient macrophage depletion in control and MSC-treated groups (73% and 50%) (Fig. 3). Infarcted mice with intact macrophages experienced lower mortality, with and without MSC transplantation (15% and 35%) (Fig. 3). Furthermore, LV dilation was greatest and contractility was lowest in surviving animals with macrophage depletion, at days 1 and 30 after MI (Table 1). The MSC therapy partially corrected these adverse effects of macrophage depletion (Table 1). Some of the early effects of transient macrophage depletion, for example its negative effect on LV area, or the favorable effect of MSC transplantation on LV remodeling and function, declined after 30 days (Table 1). Because of the high mortality rate after a single dose of CL (effective for 3 days), we avoided continuous administration of the drug. Finally, macrophage depletion in another group of normal mice was uneventful (Fig. 3).

To determine the role of macrophage subsets in phases I and II after MI, we subjected another group of animals to MI and early and late macrophage depletion, with and without MSC therapy. Remarkably, early (4 h before MI), but not late (3 days after MI), macrophage depletion impaired LV function and increased LV dilation, at day 1 and

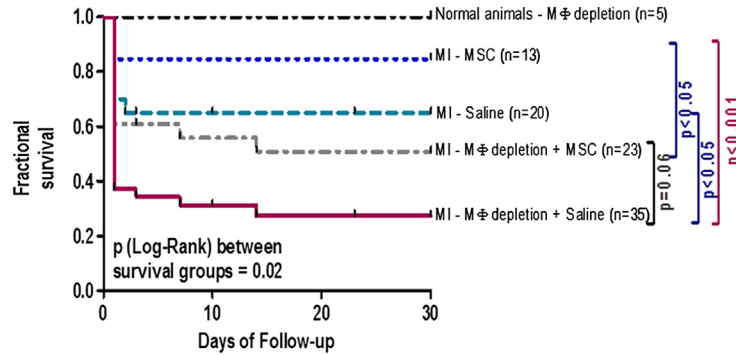


Figure 3 Macrophage Depletion Increased Mortality With and Without MSCs

Myocardial infarction (MI) after macrophage (MΦ) depletion was associated with high mortality. Mesenchymal stromal cell (MSC) therapy attenuated these negative effects. The p value is based on the log-rank (Mantel-Cox) test. Normal animals-MΦ depletion (black), n = 5; MI-MSC (blue), n = 13; MI-saline (green), n = 20; MI-MΦ depletion + MSC (gray), n = 23; MI-MΦ depletion + saline (red), n = 35.

7 after MI (Table 2). For example, cardiac contractility, presented by fractional area change and ejection fraction, was lowest after early macrophage depletion, 1 and 7 days after MI (Table 2). A week later, early depletion of macrophages increased diastolic and systolic LV areas by 30% and 68%, compared with saline (Table 2). Transplantation of MSCs, with and without macrophage depletion, attenuated adverse LV remodeling and dysfunction, 7 days after MI (Table 2).

Deterioration of global and regional myocardial function after MI and early macrophage depletion were confirmed by LV speckle-tracking based strain analysis (Table 3, Fig. 4). Early macrophage depletion decreased regional function of the infarct-related, apical and mid anterior segments (Fig. 4A), which remained low after 7 days (Table 3). Notably, MSC therapy after both early and late macrophage depletion was associated with improved regional function at the mid anterior infarct zone after 1 week (Table 3, Fig. 4B).

Interestingly, echocardiography studies, 1 week after MI, revealed large LV thrombus after early but not late macrophage depletion (Fig. 5A). This unusual finding was

confirmed by histological analysis (Fig. 5B). Significantly, MSC therapy reduced the incidence of LV thrombus, compared with control (1 of 7 vs. 5 of 6 animals; p = 0.03).

The imaging results were confirmed by histological analysis. Masson's-Trichrome staining revealed that MSCs reduced infarct size by 40%, compared with saline (Fig. 6A). Significantly, early macrophage depletion, with and without MSC therapy, was associated with the largest infarct size (Fig. 6B). Infarct vascularization, as indicated by CD31 and α -smooth muscle actin immunostaining 7 days after MI, was more than 2-fold greater after early versus late macrophage depletion (Online Fig. 4). This finding is probably related to the number of macrophages with well-recognized angiogenic properties at the time of evaluation. Remarkably, activity of cathepsin B, L, and S, as markers of macrophage proteolysis, was higher in saline-treated hearts with intact macrophages, than in MSC-treated hearts, 7 days after MI (Fig. 7).

Discussion

The major new finding of the present study suggests that some of the favorable effects of MSCs on the infarcted

Table 1 Left Ventricle Remodeling and Dysfunction by Echocardiography at 1 and 30 Days After Myocardial Infarction

		MSCs (n = 11)	Saline (n = 9)	MSCs+Early CL (n = 11)	Saline+Early CL (n = 8)	p Value
LV diastolic area, mm ²	Day 1 after MI	8.6 ± 0.3	8.9 ± 0.4	9.76 ± 0.4	10.5 ± 0.7	p interaction = 0.73
	Day 30 after MI	12.0 ± 1.2	10.7 ± 0.5	14.6 ± 3.2	13.2 ± 0.8	p treatment effect = 0.31
	p (paired t test)	0.033	0.003	0.15	0.034	p time effect = 0.003
LV systolic area, mm ²	Day 1 after MI	5.28 ± 0.3*	5.58 ± 0.5*	6.16 ± 0.2	8 ± 0.8	p interaction = 0.88
	Day 30 after MI	7.1 ± 0.5*	7.56 ± 0.8	7.27 ± 0.8	9.3 ± 0.9	p treatment effect = 0.004
	p (paired t test)	0.011	0.038	0.23	0.32	p time effect = 0.001
FAC, %	Day 1 after MI	38.75 ± 2.7*	37.64 ± 3.4	36.20 ± 2	24.6 ± 3.6	p interaction = 0.32
	Day 30 after MI	38.7 ± 3.7	30.9 ± 4.8	42.9 ± 6.5	30.2 ± 3.7	p treatment effect = 0.04
	p (paired t test)	0.99	0.22	0.34	0.3	p time effect = 0.6

The p values are based on 2-way repeated-measures analysis of variance. *p < 0.05 versus saline + early CL, based on Bonferroni post-test. CL = clodronate liposomes; FAC = fractional area change; LV = left ventricle; MI = myocardial infarction; MSC = mesenchymal stromal cell.

Table 2 Effect of MSC Therapy on Global LV Remodeling and Function With and Without Macrophage Depletion by Conventional Echocardiography at 1 and 7 Days After Myocardial Infarction

		MSCs (n = 6)	MNCs (n = 7)	Saline (n = 7)	MSCs+ Early CL (n = 5)	MSCs+ Late CL (n = 5)	Saline+ Early CL (n = 4)	Saline+ Late CL (n = 5)	p Value*
LV diastolic area, mm ²	Day 1 after MI	9.72 ± 0.4	9.9 ± 0.3	9.2 ± 0.6	10 ± 0.5	9 ± 0.3	10.3 ± 0.5	9.1 ± 0.7	0.28
	Day 7 after MI	10.8 ± 0.2	11.1 ± 0.3	11 ± 0.6	12.5 ± 0.8	9.6 ± 0.1	14.24 ± 1.8	10.4 ± 1	0.06
LV systolic area, mm ²	Day 1 after MI	5.9 ± 0.6	6 ± 0.38	5 ± 0.6	6.8 ± 0.4	6.2 ± 0.4	7.5 ± 0.5	5 ± 0.5	0.04
	Day 7 after MI	5.8 ± 0.4	7 ± 0.4	6.7 ± 0.5	7.3 ± 0.9	5.7 ± 0.4	11.26 ± 1.6	6.8 ± 1	0.07
FAC, %	Day 1 after MI	39.4 ± 4.2	32.6 ± 3.9	45.8 ± 6.3	35.03 ± 2.6	30.95 ± 4.5	27.47 ± 2.3	45.5 ± 3.7	0.05
	Day 7 after MI	45.39 ± 4.1	36 ± 3.9	37.72 ± 5.3	41.85 ± 4.7	41.04 ± 3.5	21.45 ± 1.8	36 ± 5	0.08
Ejection fraction, %	Day 1 after MI	51.82 ± 4.4	44.8 ± 4.2	61 ± 6.1	42.24 ± 5.6	42 ± 4.8	39.22 ± 5	54.9 ± 4.4	0.07
	Day 7 after MI	56.76 ± 4.5†	45.8 ± 2	46.4 ± 6.2	44.8 ± 2.6	50.16 ± 2.3	33.8 ± 2.5	44.4 ± 5.9	0.06

*The p values are based on Kruskal-Wallis test. †p < 0.05 versus saline + early CL at day 7 after MI, based on Dunn's multiple comparisons. Abbreviations as in Table 1.

myocardium are mediated by modulation of macrophage phenotype and function. Transplantation of MSC into the infarct decreases macrophage accumulation, and increases the percentage of reparative (M2) macrophages in the infarcted heart of mice. Furthermore, our in vitro experiments suggest a cross-talk between macrophages and MSCs, which results in augmentation of cytokine secretion. Importantly, early macrophage depletion partially reduced the favorable effects of MSC therapy after MI. Specifically, early macrophage depletion increased infarct size and mortality, accelerated adverse LV remodeling, dysfunction, and LV thrombus formation early after MI. These negative effects were attenuated by macrophage restoration and MSC transplantation. In contrast, late (>3 day) macrophage depletion was free of these negative effects. Taken together, we propose an additional pathway by which MSCs could improve infarct healing: modulation of infarct macrophage function.

Our findings are important because they provide further insight into the role of MSCs and macrophages in infarct repair. The healing process after MI is believed to take place with the transition of proinflammatory to reparative macrophages (19). Excessive activation of inflammatory monocytes and M1 macrophages worsens post-MI remodeling (2,20). In the last few years, it has been recognized that MSCs are modulators of immune response (5), and can attenuate a systemic inflammatory response in animal models of lung injury and sepsis (21). These protective effects could be mediated by macrophages.

Our work adds to and extends recent publications describing the role of monocytes and macrophages in infarct repair (8,9,18,22–25). van Amerongen et al. (22) found that macrophage depletion, in the first week after cryoinjury in mouse, markedly increased mortality, impaired infarct healing, and accelerated adverse LV remodeling by post-mortem histopathology. Nahrendorf et al. (8) showed that 2 distinct subsets of monocytes, the precursors of tissue macrophages, participate in healing after MI in a sequential manner. The CD11b^{high}/Ly-6C^{high} cells, exhibiting phagocytic and proinflammatory functions, accumulate during phase I (1 to 3 days), and CD11b^{high}/Ly-6C^{low} cells, attenuating an inflammatory response and expressing vascular endothelial growth factor, are present during phase II (4 to 7 days) (8).

Rapid monocyte turnover in the infarcted myocardium has been reported. Leuscher et al. (24) found that within days after injury, monocytes were recruited at a high rate but resided only for an average of 20 h in the infarcted heart. Thereafter most of the infiltrating monocytes become apoptotic. Thus, soon after MI, Ly-6C^{high} monocytes infiltrated the infarct in large numbers (8,24). Many of these monocytes do not necessarily differentiate to macrophages, but rather exit or die in the tissue. Those that differentiate acquire M1-like properties, continue to express Ly-6C, and contribute to inflammation. Over time, as inflammation gives way to resolution, a second Ly-6C^{int/low} phase emerges

Table 3 Effect of MSC Therapy With and Without Macrophage Depletion on Regional and Global LV Function by Speckle-Tracking Based Strain Imaging in Parasternal Long-Axis View

		MSCs	Saline	MSCs+ Early CL	MSCs+ Late CL	Saline+ Early CL	Saline+ Late CL	p Value*
Peak longitudinal strain		(n = 6)	(n = 6)	(n = 5)	(n = 5)	(n = 4)	(n = 5)	
Apical anterior, %	Day 1 after MI	-11.7 ± 2.5	-11.4 ± 3.5	-4.8 ± 1.7	-10.65 ± 2.6	-8.18 ± 3.7	-16.5 ± 2.4	0.08
	Day 7 after MI	-7.7 ± 1.9	-11.4 ± 3.2	-4.7 ± 1.2	-15.4 ± 4.4	-4.2 ± 2.3	-17.81 ± 2.5	0.026
Mid anterior, %	Day 1 after MI	-11.3 ± 1.8	-11.1 ± 1.6	-4.7 ± 1.5	-8.6 ± 1.6	-4.8 ± 0.9	-11.7 ± 1.5	0.01
	Day 7 after MI	-9.3 ± 1.3	-7.8 ± 1.6	-6.4 ± 1.5	-12.05 ± 1.8	-7.1 ± 1.3	-9.56 ± 1.7	0.2
Basal anterior, %	Day 1 after MI	-12 ± 2.8	-13.9 ± 4.2	-5.3 ± 2.15	-7.5 ± 2.7	-11.7 ± 4.4	-8 ± 2.1	0.3
	Day 7 after MI	-6.88 ± 2.2	-4.2 ± 1.6†	-15.6 ± 1.4	-7.9 ± 1.7	-11.44 ± 3.2	-11.56 ± 4.8	0.02
Global, ‡ %	Day 1 after MI	-14 ± 1.2	-15 ± 1.3†	-7 ± 1	-11.5 ± 1.4	-9.1 ± 2.4	-14.4 ± 2	0.01
	Day 7 after MI	-10.4 ± 1.5	-11.4 ± 2.5	-13.8 ± 1.6	-14.5 ± 1.8	-9.9 ± 1.2	-16 ± 2.9	0.2
Peak radial strain		(n = 6)	(n = 7)	(n = 5)	(n = 5)	(n = 5)	(n = 6)	
Apical anterior, %	Day 1 after MI	22.52 ± 4	20 ± 4.6	9.1 ± 2.9	22.38 ± 5	17.64 ± 10	35 ± 7.2†	0.08
	Day 7 after MI	17.54 ± 4	22.2 ± 5.55	7.4 ± 3	34.26 ± 11	9.8 ± 4.2	24 ± 5.8	0.07
Mid anterior, %	Day 1 after MI	23.25 ± 4.6	34 ± 5.3	14.46 ± 2.5	16.58 ± 2.7	10 ± 2.2	37.22 ± 5§	0.007
	Day 7 after MI	11.8 ± 4.5	16.75 ± 4.5	19.4 ± 3.3	21.65 ± 3.9	11.5 ± 2.78	14.34 ± 3.7	0.5
Basal anterior, %	Day 1 after MI	28 ± 10.37	41 ± 0.12	30 ± 8	32 ± 9.3	18.91 ± 5	20.4 ± 2.1	0.8
	Day 7 after MI	13.7 ± 6	16.7 ± 6.2	36.95 ± 7.1	18.58 ± 5	24.62 ± 11	15.11 ± 4.5	0.19
Global, ‡ %	Day 1 after MI	28.8 ± 3.5	27.9 ± 3.5	20.6 ± 3.4	34.41 ± 5	16.4 ± 3.9	34.08 ± 4.4	0.04
	Day 7 after MI	17.11 ± 3	23.6 ± 4	28.73 ± 3.8	36.38 ± 7	18.3 ± 1.8	26.96 ± 3.4	0.07

*The p values are based on Kruskal-Wallis. †p < 0.05 versus MSCs + early CL, based on Dunn's post-test. ‡Whole left ventricle average strain. §p < 0.05 versus saline + early CL, based on Dunn's post-test. Abbreviations as in Table 1.

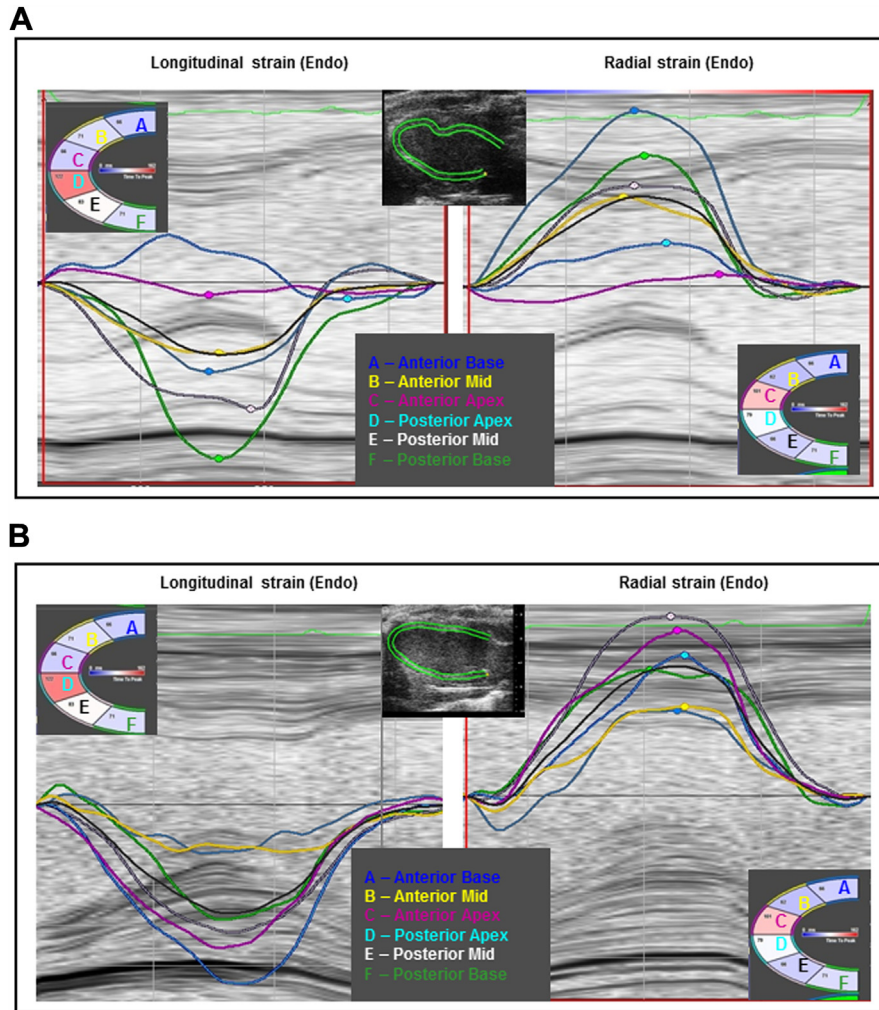


Figure 4 Representative Abnormal Apical-Anterior Strain Curves After MI and Macrophage Depletion

(A) Longitudinal and radial strain curves after myocardial infarction (MI) and early macrophage depletion show abnormal regional function and synchronization. **(B)** Longitudinal and radial strain curves after MI, mesenchymal stromal cell (MSC) therapy, and late macrophage depletion show improved regional function and synchronization. Endo = endocardial.

(8,24). The present study suggests that MSCs could affect the kinetics of a macrophage subset after MI.

Nahrendorf *et al.* (8) also reported high cathepsin activity among a Ly-6C^{hi} subset. Our study expands upon these findings and shows that MSCs increased the number of reparative macrophages and decreased activity of cathepsin B, L, and S in the infarct. Cathepsins have significant extracellular matrix proteolytic activity and can accelerate adverse LV remodeling (26). Thus, attenuation of cathepsin activity could be another pathway by which MSCs improve LV remodeling and contractility after MI.

Another striking observation was LV thrombus formation after early macrophage depletion and MI. The mechanism of LV thrombus formation is unclear. Franz *et al.* (23) who was the first to describe LV thrombus complicating macrophage depletion in acute MI, have suggested that LV

thrombus could be related to a defect in infarct healing that exposes damaged tissue to the blood. We found, for the first time, that MSC therapy markedly reduced the incidence of LV thrombus after macrophage depletion, suggesting that the development of LV thrombus is related to either extensive MI or impaired healing, or both.

An alternative mechanism is MSC fibrinolytic and antithrombogenic properties. Neuss *et al.* (27) have shown a high intrinsic fibrinolytic capacity of hMSCs that mediates the invasion into a fibrin clot of a wounded tissue. Agis *et al.* (28) have suggested that activated platelets can enhance the plasminogen activation capacity of mesenchymal progenitors through the stimulation of uPA production. Hashi *et al.* (29) have described attenuation of platelet aggregation by MSCs. Thus, the low incidence of LV thrombus after MSC transplantation could be

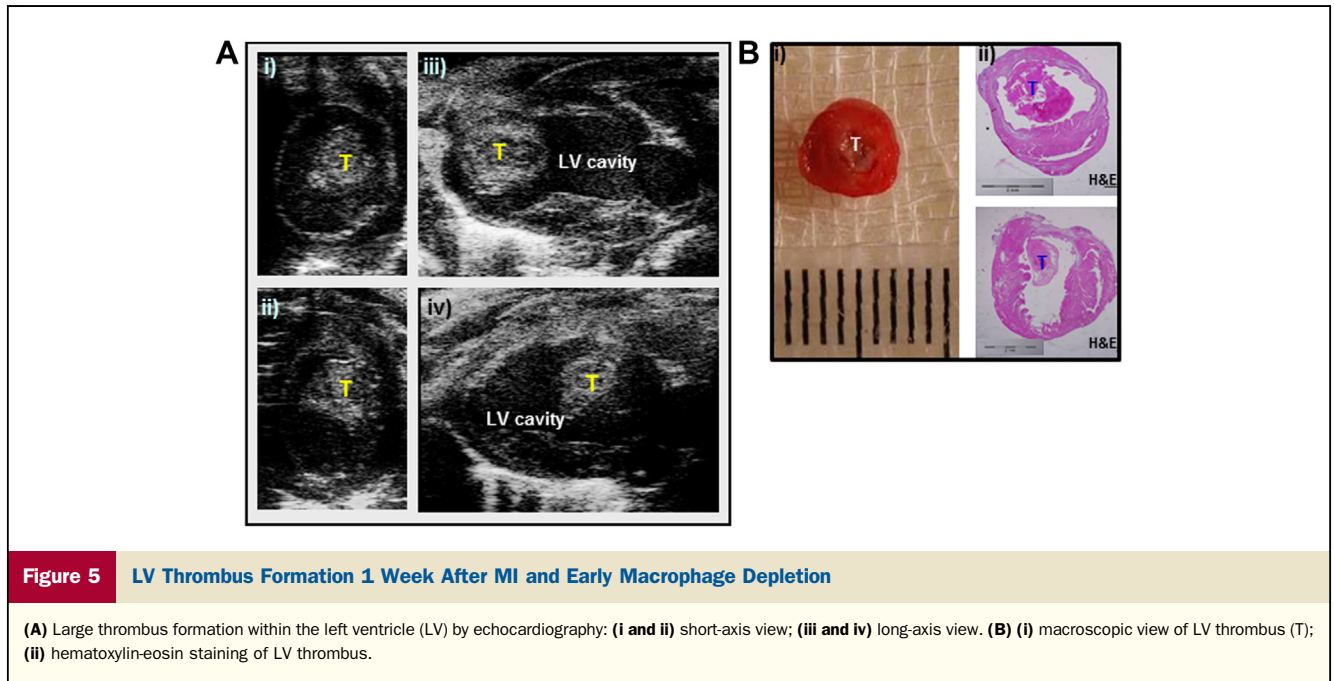


Figure 5 LV Thrombus Formation 1 Week After MI and Early Macrophage Depletion

(A) Large thrombus formation within the left ventricle (LV) by echocardiography: (i and ii) short-axis view; (iii and iv) long-axis view. (B) (i) macroscopic view of LV thrombus (T); (ii) hematoxylin-eosin staining of LV thrombus.

mediated by direct antithrombogenic properties of the implanted cells.

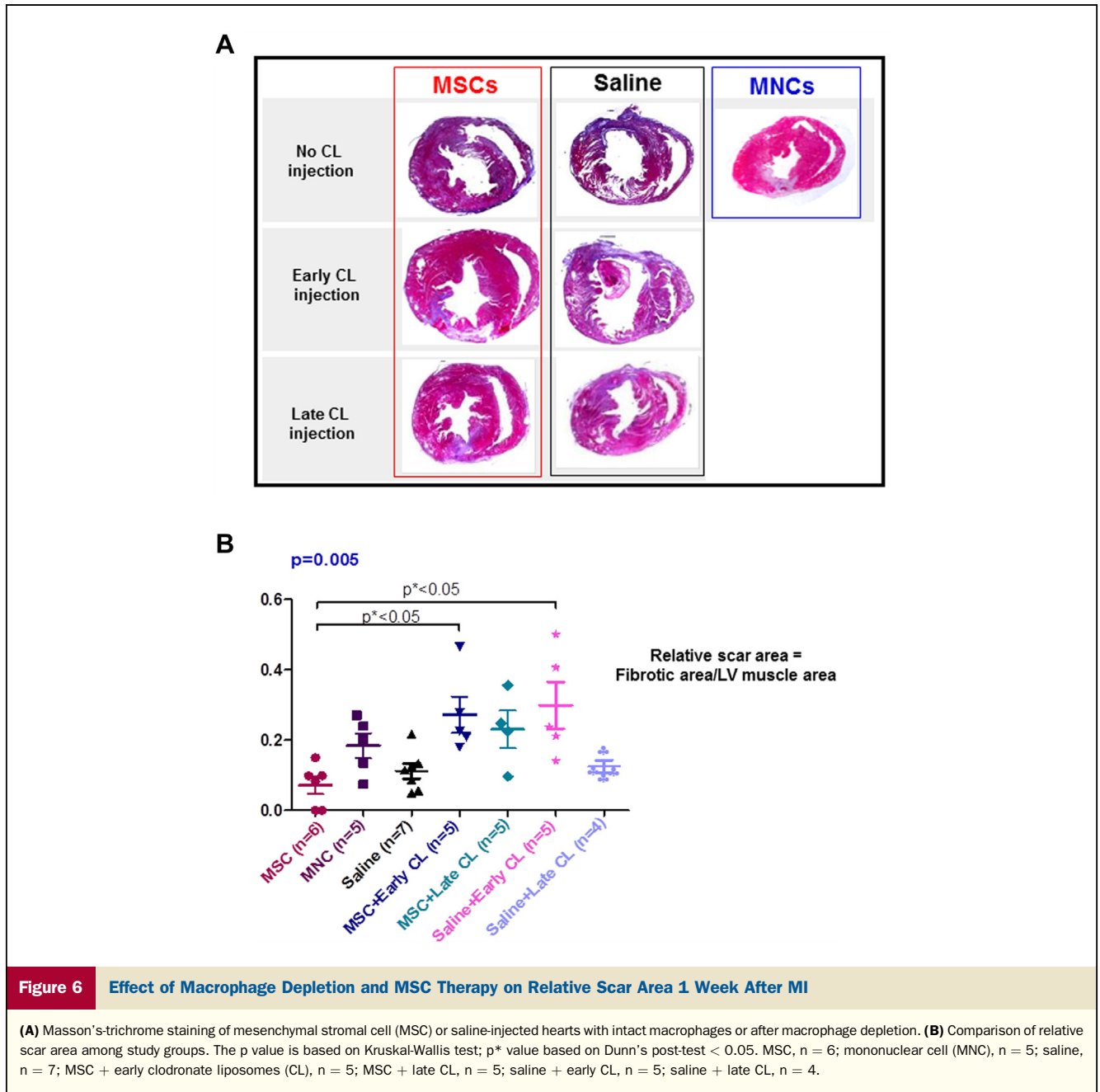
Another potential pathway by which MSCs could correct the harmful consequences of macrophage depletion, is transdifferentiation: implanted MSCs could convert into macrophages that restore healing and repair. For example, Freisinger *et al.* (30) have described the potential of adipose-tissue resident MSCs to generate functional macrophages *in vitro*. Thus, theoretically MSCs could replace the apoptotic macrophages and improve healing and repair.

Our results on the effect of MSCs on infarct macrophage modulation support and extend recent findings of Dayan *et al.* (18). They investigated the effect of 2 types of MSCs, human BM-derived MSCs and human umbilical cord perivascular cells, in an experimental MI model of the immune-deficient NOD/SCID mouse. Cells were infused 48 h after induction of MI, and mice assessed 24 h later (72 h after MI) for BM, circulating, and cardiac tissue-infiltrating monocytes/macrophages. Similar to our findings, they found that MSCs reduced the number of macrophage/monocytes, whereas the proportion of M2 macrophages was increased in the circulation and heart. Moreover, similar to our findings, fractional shortening was improved 2 weeks after cell infusion, but was similar to medium controls 16 weeks after MI. Our work confirms and adds to these findings in immune competent mouse by showing that early macrophage depletion clearly attenuates the therapeutic effects of MSCs. Additionally, we used speckle-tracking based strain imaging to assess cardiac function. Compared with conventional echocardiographic imaging, speckle-tracking based strain echocardiography efficiently detects delicate regional changes in cardiac

performance after MI and also identifies early differences in response to treatment (31).

The decline in the favorable effects of MSCs over time might be related to either massive cell loss within 7 days after transplantation (4) or to intensive monocyte and macrophage turnover in the infarcted tissue (24), or to both. Together, the favorable effects of implanted MSCs are self-limited and the disappearance of MSCs and “educated” monocytes/macrophages from the infarcted heart reduced the beneficial effect over time.

Mechanism of macrophage polarization by MSCs. Despite considerable attention, the specific molecular and cellular mechanisms involved in macrophage polarization by MSCs remain unclear. There is evidence that the ability to modulate macrophage function relies on cells, contact-dependent mechanisms, and paracrine effects through the release of soluble factors (reviewed by Delarosa *et al.* [32], Doorn *et al.* [33], and Krampera *et al.* [34]). First, MSCs need to be “primed” by inflammatory cytokines to become immunosuppressive (34). Then, it has been suggested that a broad panel of molecular pathways are involved in MSC-mediated macrophage regulation, including interferon- γ , IL-1 β , indoleamine-2,3-dioxygenase, IL-4, IL-6, IL-10, IL-13, prostaglandin-E2, TNF- α , nitric oxide, heme oxygenase-1, hepatocyte growth factor, transforming growth factor- β 1, HLA-G5, as well as many other factors, some of which are still unidentified (34). More recently described molecules with immunoregulatory functions include CCL2, galectin-1, and TNF- α -stimulated gene/protein-6. For example, expression of TNF- α -stimulated gene/protein-6 was highly upregulated after infusion of MSCs in mice with MI, and small interfering RNA (siRNA)



against it markedly reduced the beneficial effects of infused MSCs on infarct size and heart function (35). Additionally, MSCs may also modulate macrophage function through the generation of regulatory T cells.

Finally, apoptosis of transplanted MSCs could account for macrophage polarization (36). Apoptotic cell ingestion by macrophages induces expression of anti-inflammatory cytokines, such as IL-10 and transforming growth factor- β , and could suppress synthesis of proinflammatory cytokines in the infarcted heart (36). Indeed, we have shown that intravenous infusion of apoptosis-mimicking liposomes modulates macrophage phenotype and function, and improves angiogenesis and infarct repair in rat (37).

Thus, apparently, the mechanism responsible for macrophage regulation by MSCs is still unclear. Moreover, differences in the mechanisms of immunomodulation employed by MSCs from different species have been reported (32).

Study limitations. First, cardiac macrophages isolated from infarcted hearts were cultured in a purity of 80%. The F4/80 negative cells in culture are most likely fibroblasts that could affect the secretome. To adjust for this potential bias, we compared infarct macrophages from saline-treated versus MSC-treated hearts. Second, the high mortality rate after early macrophage depletion and the relatively small number of animals in certain experiments could create a selection bias. Finally, the differences among groups were tested by several

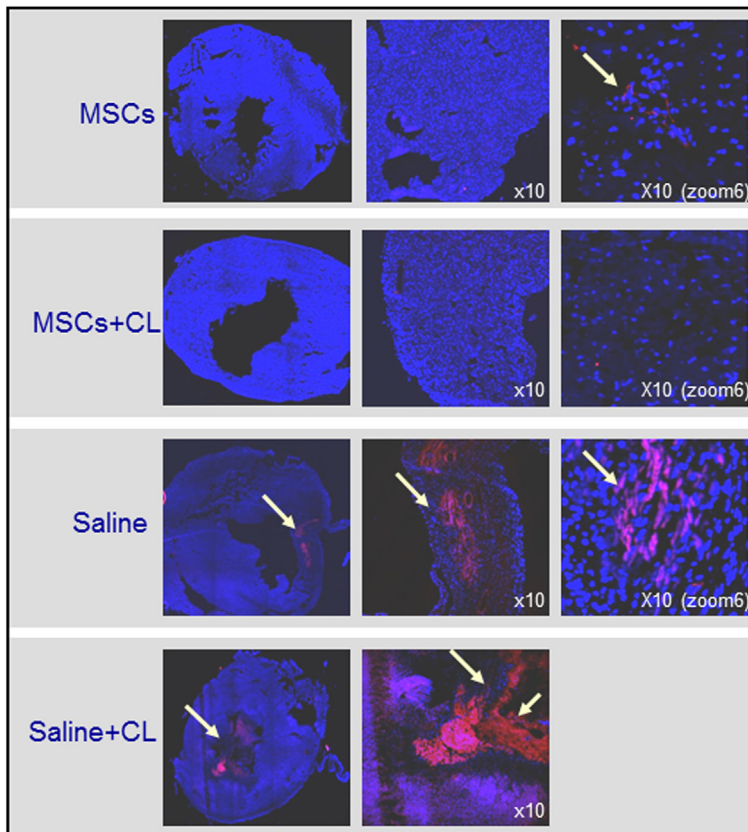


Figure 7 MSC Inhibited Cathepsin Activity 1 Week After MI

Hearts were cryosectioned and labeled for cathepsin activity. Positive activity was detected by red fluorescent color (**arrows**). CL = clodronate liposomes; MI = myocardial infarction; MSC = mesenchymal stromal cell.

parameters. This could increase the risk of type I error (false positive).

Conclusions

Our study emphasizes the critical role of monocyte/macrophage modulation in infarct repair, and proposes an additional pathway by which MSCs could improve infarct healing: MSCs can act specifically at the macrophage level. The importance of these findings lies in the fact that targeting macrophages by MSCs or other means could be a novel approach to ameliorate tissue injury not only in the setting of MI but also in other diseases. Understanding the signaling pathways and the fate of monocytes and macrophages after MI will support the search for specific therapies. For example, intravenous infusion of apoptosis-mimicking liposomes modulates macrophage phenotype and function, and improves angiogenesis and infarct repair in rat (37). Alternatively, silencing the chemokine receptor CCR2 gene by siRNA inhibits the recruitment of inflammatory monocyte subset in mice with MI, attenuates infarct inflammation, and limits post-MI LV remodeling (38).

Taken together, the ability to improve infarct healing and repair by monocyte/macrophage modulation could advance the field of cardiovascular regenerative medicine, particularly in elderly and sick persons who have impaired immune systems and regenerative potential.

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Key Words: inflammation ■ macrophage ■ mesenchymal stroma cell ■ myocardial infarction ■ remodeling.

▶ APPENDIX

For an expanded methods section, as well as supplemental tables and figures, please see the online version of this article.