Intravenously Delivered Mesenchymal Stem Cells
Systemic Anti-Inflammatory Effects Improve Left Ventricular Dysfunction in Acute Myocardial Infarction and Ischemic Cardiomyopathy

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Rationale: Virtually all mesenchymal stem cell (MSC) studies assume that therapeutic effects accrue from local myocardial effects of engrafted MSCs. Because few intravenously administered MSCs engraft in the myocardium, studies have mainly utilized direct myocardial delivery. We adopted a different paradigm.

Objective: To test whether intravenously administered MSCs reduce left ventricular (LV) dysfunction both post–acute myocardial infarction and in ischemic cardiomyopathy and that these effects are caused, at least partly, by systemic anti-inflammatory activities.

Methods and Results: Mice underwent 45 minutes of left anterior descending artery occlusion. Human MSCs, grown chronically at 5% O₂, were administered intravenously. LV function was assessed by serial echocardiography, 2,3,5-triphenyltetrazolium chloride staining determined infarct size, and fluorescence-activated cell sorting assessed cell composition. Fluorescent and radiolabeled MSCs (1×10⁶) were injected 24 hours post–myocardial infarction and homed to regions of myocardial injury; however, the myocardium contained only a small proportion of total MSCs. Mice received 2×10⁶ MSCs or saline intravenously 24 hours post–myocardial infarction (n=16 per group). At day 21, we harvested blood and spleens for fluorescence-activated cell sorting and hearts for 2,3,5-triphenyltetrazolium chloride staining. Adverse LV remodeling and deteriorating LV ejection fraction occurred in control mice with large infarcts (225% LV). Intravenous MSCs eliminated the progressive deterioration in LV end-diastolic volume and LV end-systolic volume. MSCs significantly decreased natural killer cells in the heart and spleen and neutrophils in the heart. Specific natural killer cell depletion 24 hours post–acute myocardial infarction significantly improved infarct size, LV ejection fraction, and adverse LV remodeling, changes associated with decreased neutrophils in the heart. In an ischemic cardiomyopathy model, mice 4 weeks post–myocardial infarction were randomized to tail-vein injection of 2×10⁶ MSCs, with injection repeated at week 3 (n=16) versus PBS control (n=16). MSCs significantly increased LV ejection fraction and decreased LV end-systolic volume.

Conclusions: Intravenously administered MSCs for acute myocardial infarction attenuate the progressive deterioration in LV function and adverse remodeling in mice with large infarcts, and in ischemic cardiomyopathy, they improve LV function, effects apparently modulated in part by systemic anti-inflammatory activities.

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Key Words: cardiomyopathies ■ inflammation ■ killer cells, natural ■ myocardial infarction ■ stem cells

Although percutaneous coronary intervention has improved outcomes for patients with acute myocardial infarction (AMI), some patients with AMI, including those with minimal evidence of left ventricular (LV) dysfunction, develop progressive adverse LV remodeling despite optimal medical therapy.¹ Importantly, after AMI, the immune response, particularly the innate response, plays a crucial role in both myocardial recovery and remodeling processes.² We and others suggested that
Novelty and Significance

What Is Known?

- Stem cell therapeutic efficacy has been thought to depend on local myocardial effects, necessitating efficient myocardial engraftment.
- Intravenous delivery of stem cells results in low cardiac engraftment leading to a focus on catheter-based delivery.
- Continuing inflammation probably contributes to progressive myocardial dysfunction occurring in patients after a myocardial infarction and in patients with ischemic or nonischemic cardiomyopathy.

What New Information Does This Article Contribute?

- Intravenous administered mesenchymal stem cells (MSCs) inhibit progressive myocardial deterioration that occurs after myocardial infarction and during chronic heart failure.
- The improved outcomes of cell therapy seem to be related to potent systemic anti-inflammatory effects.
- Stem cells do not have to be delivered directly to the heart to improve cardiac function.

It is generally assumed that for stem cells to improve myocardial function in patients, large numbers of cells need to engraft in the myocardium to exert local effects. Because intravenous delivery of MSCs leads to sparse myocardial engraftment, focus has been almost exclusively on catheter delivery of cells, an impractical strategy for repeated administrations. Chronic inflammation seems to contribute to progressive myocardial deterioration that occurs after infarction and in patients with heart failure. Because MSCs secrete factors with multiple activities, including anti-inflammatory effects, we postulated that intravenously administered MSCs could improve clinical outcomes, in part, by systemic anti-inflammatory effects. MSCs were injected intravenously into mice with myocardial infarction or with ischemic cardiomyopathy. Marked improvements in left ventricular function occurred, which were associated with anti-inflammatory effects. An MSC-induced decrease in NK cells seemed to play an important role in their beneficial cardiac effects. This study shows that despite low myocardial engraftment, intravenously administered MSCs improve cardiac function in both acute myocardial infarction and ischemic cardiomyopathy, effects modulated in part by systemic anti-inflammatory effects.

Nonstandard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
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<td>LV</td>
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<td>LVEDV</td>
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<td>MI</td>
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<td>MSC</td>
<td>mesenchymal stem cell</td>
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These same proinflammatory immune responses, essential for myocardial healing, may be excessive and thereby constitute a major cause of progressive adverse remodeling and myocardial dysfunction. A similar evolution has occurred in our concepts relating to the mechanisms responsible for progressive LV dysfunction in patients with chronic ischemic or nonischemic cardiomyopathy. Thus, excessive immune/inflammatory responses may importantly contribute to these patients’ progressive myocardial dysfunction.

This inflammatory concept of disease progression has led us to explore the potential of a particular strategy that has the capacity not only to modulate inflammatory responses but also to favorably alter other mechanisms thought to contribute to progressive LV dysfunction. That strategy is the systemic administration of mesenchymal stem cells (MSCs).

Our focus on MSCs derives from coupling the inflammatory hypothesis of progressive cardiac dysfunction post-AMI and in patients with cardiomyopathy, with known activities of MSCs. Among these are their immunomodulatory effects, leading to suppression of both innate and adaptive immune responses. In addition, the ability of MSCs to secrete a broad array of factors influencing, for example, adverse LV remodeling, angiogenesis, tissue healing, apoptosis, mitochondrial dysfunction, microvascular dysfunction, and collagen deposition raises the possibility that their multitarget functionality could, separately and synergistically, increase the likelihood of this strategy leading to clinically important inhibition of adverse LV remodeling and progressive deterioration of LV function.

Another consideration of major practical consequence is the concept, driving the design of most preclinical and clinical studies, that any beneficial effect of MSCs derives from their local effects. It is commonly thought that once engrafted in damaged myocardium, stem cells either directly transdifferentiate into functional myocardium, stimulate resident myocardial stem cells to expand and repopulate the heart with functioning myocytes, or secrete substances leading to myocardial repair. This orientation, along with the demonstration that stem cells, administered intravenously, only poorly engraft in ischemic myocardium, led to reliance on intracoronary, transendocardial, or intramyocardial administration of stem cells.

Our strategy for stem cell administration was based on a very different paradigm. If an excessive and prolonged inflammatory process is a key player in causing progressive cardiac dysfunction, then it is possible that MSCs, administered intravenously, might exert through paracrine mechanisms systemic anti-inflammatory effects. Intravenous delivery would have an additional advantage considering that the chronic processes leading to progressive LV dysfunction will probably never be cured by a single administration of stem cells. If true, then repeated injections over time would be necessary for a sustained therapeutic effect—a situation in which the intravenous route of administration would have a huge advantage over catheter-based delivery strategies.

We tested these concepts in studies on murine AMI and ischemic cardiomyopathy models. We used standard ischemia/reperfusion models of AMI (to simulate the clinical condition that would exist in patients undergoing urgent percutaneous coronary intervention for AMI) and of ischemic cardiomyopathy. MSCs were obtained from a young healthy human donor.
We expanded the MSCs under chronic hypoxic conditions, similar to their natural niche in the bone marrow, as exposure of MSCs to these growth conditions enhances their functionality.\textsuperscript{23} Of relevance, hypoxia-preconditioning of cardiac progenitor cells seems to improve cardiac outcome in experimental AMI.\textsuperscript{24} Thus, this study explores the efficacy of intravenously administered human MSCs, grown under chronic hypoxic conditions, on (1) LV function after AMI, (2) LV function in ischemic cardiomyopathy, and (3) whether MSC-induced beneficial effects derive, at least in part, from their immunomodulatory activities.

**Methods**

**Ethics Statement**
Animal handling and care followed the recommendations of the National Institute of Health guide for care and use of laboratory animals. All protocols were approved by the animal care and use committee at Washington Hospital Center, Washington, DC.

**Animals**
All animals used in the current study were male CD1 mice. Mice were 8 to 10 weeks of age with body weight of 25 to 35 g.

**AMI Model**
AMI was produced by temporary ligation of the left anterior descending coronary artery for 45 minutes followed by reperfusion. Ligation was considered successful when the anterior wall of the LV turned pale (Online Methods). After AMI, mice were individually housed. The top panel of Online Figure I depicts a schematic of the experimental protocol.

**Human MSCs**
Primary frozen bone marrow–derived human MSCs were received from Stemedica (San Diego, CA). Culture conditions are described in Online Methods. Unless otherwise specified, 2×10\textsuperscript{6} MSCs in 0.2 mL were injected in each mouse into the lateral tail vein 24 hours after AMI (Online Methods).

**Radiolabeled MSC Experiments**
Cells were labeled with indium-111 oxine. Tail-vein injection of 1.0×10\textsuperscript{6} radiolabeled MSCs was performed 24 hours after AMI (n=10) and in control mice without myocardial infarction (MI; n=10; Online Methods). Approximately 20 hours after intravenous injection of radiolabeled MSCs, organs were harvested. Gamma well counting and phosphor imaging (Perkin Elmer) was performed to assess radioactivity levels within the organs. Data were analyzed in a blinded fashion (Sante DICOM software) with adjustment for the injected radioactive dose per animal and background.

**Ex Vivo Fluorescent Imaging**
MSCs were labeled fluorescently with Qdot 705 (Invitrogen) or ICG 820 (Sigma) and injected as above. To identify tissue-engrafted MSCs, mice were euthanized and organs were harvested and analyzed using the In Vivo Imaging System (IVIS) 100 (Perkin Elmer) with the relevant channel.

**Ischemic Cardiomyopathy Model**
Four weeks after AMI, mice were randomized based on their LV ejection fraction (EF) to either the MSC therapy group (n=16) or PBS vehicle control injection group (n=16). Thus, mice in each group will have comparable baseline LVEF before treatment. Mice then underwent tail-vein injection and received either 2×10\textsuperscript{6} MSCs in 0.2 mL of PBS or 0.2 mL of PBS control. After serial echocardiography at weeks 1 and 3, mice received a repeat treatment after the week 3 echocardiogram. After echocardiography at weeks 4 and 6, mice were euthanized after week 6 echocardiography and blood, heart, and spleen were harvested for analysis. The bottom panel of Online Figure I depicts a schematic of the experimental protocol.

**Echocardiograms**
Echocardiography (VEVO 1100 Imaging system; VisualSonics Inc) was performed under light anesthesia. Short-axis images were acquired in a blinded fashion with corresponding M-mode imaging. M-mode images were utilized to calculate LV functional parameters (Online Methods).

**Flow Cytometry**
Single-cell suspensions for flow cytometry were prepared. Cells surface Fc receptors were blocked, and cells were stained with the relevant antibodies and viability marker. Flow cytometry was performed on fluorescence-activated cell sorting Caliper (Becton Dickinson, CA), and analysis was performed with FlowJo software (Ashland, OR; Online Methods).

**2,3,5-Triphenyltetrazolium Chloride staining.**
After the day 20 echocardiograph, mice were euthanized and perfused by LV puncture using PBS. Short-axis cuts of the heart at 1-mm thickness were incubated at 37°C in 1% 2,3,5-triphenyltetrazolium chloride for 10 minutes. Heart sections were fixed in 2% paraformaldehyde and photographed, and infarcted area was analyzed using Image J software (NIH, Bethesda, MD; Online Methods).

**In Vivo Natural Killer Cells Depletion**
For in vivo natural killer (NK) cells depletion, the antibody anti-NK1.1 clone was injected intravenously (100 mg/mouse) 24 hours before MI surgery (n=12). Echocardiography was performed 7 and 21 days post-MI. At 21 days post-MI, infarct size (2,3,5-triphenyltetrazolium chloride) was analyzed and the ratio of spleen immune cells was analyzed by fluorescence-activated cell sorting. Additional mice were randomized to NK depletion or control and underwent AMI to determine the impact of NK depletion on the immune cell content within the spleen, heart, and blood 24 hours and 7 days after AMI (Online Methods).

**Results**

**Distribution of MSCs Administered IV**
To determine in vivo distribution, human MSCs (1×10\textsuperscript{6} per mouse; n=12), radioactively or fluorescently labeled, were administered intravenously to mice 24 hours after AMI or to control mice without AMI.

Radiolabeled human MSCs were detected in all measured organs at 24 hours post-injection (Figure 1A), showing high concentrations in the liver, spleen, and kidneys. A small portion of MSCs engrafted in the heart. There was preferential engraftment in the heart after AMI compared with hearts from animals without AMI (Figure 1B). Within MI hearts, MSCs concentrated predominantly in regions of injury (Figures 1D and 1E). Increased MI size strongly correlated with enhanced MSC engraftment in the left anterior descending territory (Figure 1F). Significantly higher MSC accumulation occurred in spleens of MI mice (Figure 1C), indicating MI generated a systemic signal, leading the spleen to express homing signals for MSC accumulation.

This experiment was also performed with radiolabeled murine MSCs (grown under chronic hypoxia); we found greater murine MSC trafficking to the heart and spleen in mice after AMI compared with mice the underwent sham surgery (Online Figure II). The data displayed in this figure also provide comparative data between in vivo distribution of intravenously administered human and murine MSCs 24 hours post-injection. There were significantly fewer human MSCs that trafficked to the heart and spleen compared with murine MSCs.
The decreased engraftment of human versus murine MSCs might be explained by our in vitro studies demonstrating complement-mediated lysis of human MSCs by murine serum (data not shown). Despite this immune response to human MSCs, we demonstrated persistent viability of human MSCs in the heart for as long as 3 weeks. Thus, fluorescently labeled MSCs injected intravenously 24 hours post-MI localized to the left anterior descending territory of the heart and were seen on fluorescent imaging 7 days post-MI (Figure 1G). Fluorescently labeled MSCs injected intravenously into mice with ischemic cardiomyopathy also localized to the left anterior descending territory as seen on fluorescent imaging 24 hours post-injection (Figure 1G). When gating on live cells (7AAD-negative cells), flow cytometry of single-cell
suspensions from the hearts demonstrated that human MSCs within the myocardium were viable 7 days after injection in the acute MI model (Figure 1H and 1I) and 3 weeks after injection in the ischemic cardiomyopathy model (see below; Figure 4A).

Effects of MSCs on LVEF and LV Volumes

Acute Myocardial Infarction
At day 21 post-MI, infarct size in the treated and control groups did not significantly differ (Figure 2A). Although LVEF, LV end-diastolic volume (EDV), and LV end-systolic volume (ESV) significantly worsened over 3 weeks, the degree of deterioration was attenuated in the MSC-treated group; however, the differences between the treated and control groups were not statistically significant (Figure 2B through 2D). In controls, the end-diastolic and end-systolic wall thicknesses of both the anterior and posterior walls thinned over the 3 weeks after AMI, changes compatible with progressive LV dilatation unaccompanied by compensatory LV hypertrophy (Online Figure III). In contrast, MSC treatment prevented significant end-systolic thinning of the both the anterior and posterior walls (Online Figure III) and prevented significant end-diastolic thinning of the posterior wall (Online Figure III). Importantly, in addition to preventing the deleterious changes occurring over time in the control group, when data in the treated and control groups were directly compared, the differences between the treated and control groups were statistically significant (Online Figure III).

Comparative Analysis of Mice With Small Versus Large Infarcts
Further analysis of cardiac function revealed that MSC treatment improved adverse LV remodeling, but these effects were limited to mice with MIs ≥25% of the LV (median infarct size was 25%). In this subgroup, MSC treatment prevented the statistically significant increases in end-diastolic and end-systolic volumes exhibited by the control group and there was a trend in preventing a decrease in LVEF (Figure 2E through 2G).

Ischemic Cardiomyopathy
One mouse in the MSC treatment group with a severely reduced LVEF died when undergoing echocardiography and tail-vein injection at week 3. As might be expected, 2 intravenous injections of MSCs in mice with ischemic cardiomyopathy did not reduce MI size compared with hearts from PBS-injected control mice (29.6±9.1% versus 26.7±9.1%, respectively; P=0.38; Figure 2H). In our model of ischemic cardiomyopathy, baseline values represent echocardiographic parameters 4 weeks post-MI. MSC treatment significantly increased LVEF from baseline, whereas there was no change in LVEF in controls (Figure 2I). Interestingly, MSC treatment significantly increased anterior and posterior wall thickness in systole compared with control (Online Figure III). Although the decrease in LVEDV with MSC treatment did not persist (Figure 2J), MSC treatment significantly reduced LVESV compared with control (Figure 2K). Thus, MSC treatment led to persistent improvements in LV contraction compared with control, reflected by significantly increased systolic wall thickness, reduced LVESV, and increased LVEF.

Immunomodulatory Effects of MSCs
We found higher engraftment of MSCs in spleens of MI versus no MI mice (Figure 1C), suggesting AMI leads to a systemic signal that recruits MSCs to the spleen. We suspected that this signal could also impact the spleen’s inflammatory milieu. Flow cytometric analysis of the spleen 21 days after AMI demonstrated that MSCs significantly reduced the percentage of splenic NK cells (Figure 3A and 3B), but did not alter other cell subtypes in either lymphocytic or myeloid compartments (Figure 3C).

To better understand the effect of MSCs on NK cells, we explored the impact of MSCs injected 24 hours post-MI on the composition of the different immune cells in the spleen at 7 days post-MI. Splenic NK cells were significantly increased in mice 7 days post-MI (Figure 3D). Similar to the 21 days post-MI study (Figure 3A and 3B), MSCs significantly reduced the ratio of splenic NK cells when administered 24 hours post-MI (Figure 3D) and did not alter the ratio of other immune cells from both the lymphocytic and myeloid compartments (data not shown). An MSC-induced decrease in NK cells was also observed in the heart (Figure 3E). To verify that the reduction in splenic NK cells is specific to the immunomodulatory effects of human MSCs and not related to a xenograft immune response, we performed a separate experiment in which human fibroblasts were injected 24 hours post-MI. When compared with control mice that underwent MI, mice injected with human fibroblast 24 hours post-MI did not experience a decrease in NK cells either in the spleen or the heart (Figure 3F).

Mechanism By Which MSCs Influence NK Cell Population
MSCs could affect NK cells through either direct cell to cell contact or secreted factors. To determine whether MSCs impact NK cells via secreted factors, we conducted an in vitro experiment using a transwell system (0.4-μm pore insert). Murine splenocytes were placed in the insert and human MSCs were placed in the wells in a gradient of 10⁴ to 100×10³ cells per well, allowing cells to incubate for 96 hours. NK suppression by MSCs showed a dose–response effect (Figure 3G). Thus, one of the mechanisms by which MSCs suppress NK cells occurs via secreted factors.

Neutrophils are known as immediate responders to injury and have been shown to be major contributors to adverse myocardial remodeling after MI. NK cells and neutrophils have previously been shown to regulate each other. In light of the ability of NK cells to regulate neutrophils, we explored whether MSC-induced NK reduction has an effect on neutrophil content in the heart. Although there was not a significant reduction of splenic neutrophils at 7 days post-MI after intravenous MSC administration (Figure 3C), we found that treatment with MSCs significantly reduced the accumulation of neutrophils in the myocardium (Figure 3H and 3I). These data suggest that human MSCs may either directly reduce myocardial NK cell and neutrophil content or the reduction in NK cells could serve to reduce neutrophil infiltration, thus modulating the inflammatory process after AML.

In the ischemic cardiomyopathy model, viable human MSCs were present in the apex of hearts from the MSC treatment group (Figure 4A), demonstrating human MSCs can remain viable for 3 weeks after injection and evade the immune system.
Figure 2. Intravenously administered human mesenchymal stem cell (MSCs) improve cardiac function in murine acute myocardial infarction (MI) and ischemic cardiomyopathy models. MSCs were administered 24 h post-MI, and their therapeutic effect was evaluated. Infarct size was calculated from 2,3,5-triphenyltetrazolium chloride (TTC)–stained heart sections (A). Echocardiography was performed at baseline before MI and then again at days 3, 7, and 20 post-MI. Comparison is provided for left ventricular ejection fraction (LVEF; B), LV end-diastolic volume (EDV; C), and LV end-systolic volume (ESV; D) for the MSC treatment vs control (Continued)
response. To assess the impact of intravenously administered human MSCs on splenic inflammatory cell content in the ischemic cardiomyopathy model, the spleens of mice were harvested and flow cytometry was performed. Comparison of different immune cell composition in the spleen between mice that received MSC treatment and PBS control is presented in the Table. Flow cytometry demonstrated that intravenously administered MSCs significantly reduced neutrophils (2.6±0.7 versus 4.9±1.3 for control; $P<0.0001$; Figure 4B and 4C). However, there were no significant differences in the percentage of monocytes (Figure 4B and 4D). Intravenous injection of MSCs significantly reduced myeloid cells compared with control (18.5±2.5% versus 24.1±4.1%, respectively, $P<0.0001$). There was also a trend toward reduction in NK cells with MSC treatment compared with control (Table; Figure 4E). With the reduction in myeloid cells, there was a resultant trend toward increased lymphocyte percentages with MSC treatment compared with control (77.9±11.0% versus 71.8±12.3%, respectively; $P=0.16$). This resulted in a significantly reduced splenic neutrophil:lymphocyte ratio for MSC treatment compared with control (0.072±0.024 versus 0.146±0.057, respectively; $P<0.0001$).

We next sought to determine whether there are associations between the altered splenic cellular populations and LV dimensions and function in the ischemic cardiomyopathy model. Although MSC therapy significantly reduced the levels of myeloid cell lines, there were no significant correlations between the percentage of splenic myeloid cells and LV dimensions or function for either treated or control mice. However, among mice that received MSC treatment, there were significant associations between higher percentages of splenic T lymphocytes, including CD3+, CD4+, and CD8+ T lymphocytes, with improved LV remodeling and function: that is, reduced LVESV, reduced LVEDV, and a greater LVEF (Online Figure IV). These associations were not observed in the control mice. Although splenic neutrophil:lymphocyte ratio was not associated with infarct size ($R^2=0.00$; $P=0.92$), neutrophil:lymphocyte ratio strongly correlated with LVEF at 6 weeks ($R^2=0.31$; $P=0.001$), a finding largely driven by the immunomodulation associated with MSC treatment.

**Causal Role of NK Cells in Deterioration of Cardiac Function Post-AMI**

We next sought to determine whether the reduction in NK cells caused by intravenously administered MSCs plays a direct causal role in the beneficial effects that MSCs exert on cardiac function post-AMI. To answer this question, we determined the effect of injecting intravenously, 24 hours before MI, an antibody that depletes NK cells. Thus, we sought to determine whether depletion of NK cells before AMI attenuated injury after AMI. In mice that underwent anti-NK1.1 antibody-mediated NK cell depletion 24 hours before AMI, infarct size 20 days post-MI was significantly smaller (Figure 5A). Furthermore, NK cell depletion before AMI significantly attenuated the deterioration seen in cardiac function after AMI. Importantly, LVEF was significantly higher and LVEDV and LVESV were significantly lower in the NK depletion group than in the control (Figure 5B through 5D).

**NK Cells Support a Proinflammatory Immune Response Post-MI**

A single intravenous injection of anti-NK1.1 antibody 24 hours before MI maintained significantly lower splenic NK cells at 20 days post-MI (Figure 5E and 5F). There was not a significant effect on other immune cells in the lymphocytic and myeloid compartments (Figure 5G). To determine how NK cell depletion resulted in significant cardioprotection after AMI, we assessed the impact of NK cell depletion on the spleen and heart 24 hours and 7 days post-MI. NK cell depletion significantly reduced splenic neutrophil content 24 hours post-MI, an effect that no longer remained significant 7 days post-MI (Figure 6A and 6B). As expected with resolution of the inflammatory response post-MI, there was a significant reduction in splenic neutrophils at 7 days compared with 24 hours post-MI (Figure 6B). Interestingly, there was not a significant difference in splenic monocytes at either 24 hours or 7 days post-MI (Figure 6A and 6C). Within the heart, NK cell depletion significantly reduced neutrophil infiltration 24 hours post-MI (Figure 6D and 6E). However, there was no longer a significant difference in myocardial neutrophils at 7 days post-MI. Interestingly, there was a significant increase in myocardial monocytes at 24 hours and 7 days post-MI in the NK depletion group (Figure 6F).

**Discussion**

Most previous stem cell studies have assumed that the underlying mechanism by which stem cells might exert beneficial therapeutic effects in AMI or chronic cardiomyopathy derive from local effects of stem cells that have engrafted in the injured myocardium.15-17 Because low numbers of stem cells engraft in the myocardium after intravenous administration, attention has focused, with rare exception,15-17,28 on refining direct delivery of cells to the myocardium via catheter-based delivery of cells.29-31

The current study design derives from several concepts. First, that a persistent excessive inflammatory response exists that probably contributes importantly to progressive myocardial dysfunction both in post-AMI and in cardiomyopathy patients.3,4,32 Second, these chronic inflammatory responses probably cannot be permanently suppressed by a single injection of a therapeutic. Thus, repeated injections will be necessary, a situation that would make catheter-based delivery systems problematic. Third, MSCs secrete multiple factors with a large array of activities, including anti-inflammatory
Figure 3. Mesenchymal stem cells (MSCs) exert suppressive effects on natural killer (NK) cells and neutrophils. MSCs were administered 24 h post-MI. The ratio of spleen NK cells was analyzed by fluorescence-activated cell sorting (FACS) at 21 d post-MI (A and B) as well as other immune cells from lymphocyte and myeloid compartments (C; n=15 per group). The effect of MSCs on spleen and heart NK cells was explored at day 7 post-MI by FACS analysis (D and E; n=8). Similarly, as a comparison to MSCs, the effect of human fibroblasts on spleen and heart NK cells was tested by FACS analysis 7 d post-MI (F; n=7 per group). Effects of (Continued)
effects. It therefore seems possible for beneficial cardiac effects to accrue from not only MSCs engrafted locally in the myocardium but also systemic anti-inflammatory actions of products secreted by MSCs located in tissues other than the myocardium.

This provided a major rationale for our testing the therapeutic efficacy of MSCs administered intravenously despite low myocardial engraftment. We only found 2 previous publications, both preclinical, examining the effects of IV MSCs on cardiac function and inflammation. Both demonstrated improved cardiac outcomes and, importantly, seemed to show that MSCs exerted anti-inflammatory effects. Interpretation of these 2 studies, however, is complicated by the fact that the investigators infused human MSCs into immunocompromised (NOD/SCID [nonobese diabetic/severe combined immunodeficiency]) mice, leaving uncertain what the immunomodulatory and salutary cardiac effects of the MSCs would be in an immunocompetent animal.

Our initial experiments were designed to determine the fate of human MSCs when administered intravenously 24 hours post-AMI. Similar to previous reports, we demonstrated that intravenously administered MSCs distributed to multiple organs, but only a small portion homed to myocardium (Figure 1A)—these engrafted preferentially in ischemic myocardium (Figure 1D and 1E). The myocardial-engrafted cells persisted (Figure 1G) and were viable for at least 7 days (Figure 1H and 1I). The number of engrafted cells directly related to the magnitude of LV infarct (Figure 1F). We also demonstrated that human MSCs injected intravenously into mice with ischemic cardiomyopathy engrafted in the myocardium (Figure 1G) and persisted in a viable state for at least 3 weeks (Figure 4A). MSC numbers were also significantly higher in spleens of mice with MI than those of naive mice (Figure 1C), demonstrating myocardial injury generates systemic signals that drive the cardiosplenic axis and recruit MSCs.

**Effects of IV MSC Administration in Mice With AMI**

Our experiments demonstrated that infarct size was unchanged after MSC administration (Figure 2A). Furthermore, we found that LV functional deterioration after AMI is not a process completed after a few days. Rather, both adverse LV remodeling (characterized by increasing LVEDV and LVESV) and decreasing LVEF continue over the first few weeks post-AMI (Figure 2B through 2D, control mice). There was a trend suggesting that MSCs reduce this progressive deterioration (Figure 2B through 2D). These positive trends led us to examine the hypothesis that there might be greater adverse LV remodeling in mice with large versus small infarcts, and if there was a benefit from MSC treatment, it would likely be limited to the large infarct group. This speculation proved correct. Treatment with MSCs prevented cardiac deterioration in mice with large infarcts, maintaining levels of LVEF, LVEDV, and LVESV at values similar to those of mice with small infarcts (Figure 2E through 2G). Thus, even though most MSCs administered intravenously reside in tissues other than the myocardium, intravenously administered MSCs prevent the adverse remodeling and deterioration in LV function occurring >3 weeks after AMI in mice with large infarcts.

The immunomodulatory capacity of MSCs has been reported extensively. We sought to determine whether the beneficial myocardial effects of MSCs are mediated, at least partly, by modulating the inflammatory response triggered by acute ischemic injury. We initially explored possible associations between MSC administration and changes in subpopulations of immune cells. Increasing data suggest the existence of a functional cardiosplenic axis, in which the spleen alters its immune/inflammatory cell populations in response to signals produced by injured tissue, leading to mobilization of immune/inflammatory cells that then home to the injured tissue.

At 7 days post-AMI, the percent of NK cells in the spleen is significantly increased (Figure 3D). Treatment with MSCs significantly reduced splenic NK cells to levels of naive mice (Figure 3D). There was also a significant reduction in cardiac NK cells with MSCs (Figure 3E). We demonstrated that this effect was not because of use of a xenograft model, as administering human fibroblasts did not alter splenic or cardiac NK cell populations after AMI (Figure 3F). Figure 4E demonstrates the serial changes observed in NK cell levels present in the spleen over the time course encompassed by the acute through the ischemic cardiomyopathy studies. NK cells increase over the first week post-AMI and then gradually decline. MSCs were able to maintain a lower NK cell level throughout the period of the experiments.

Previous in vitro studies demonstrated that MSC-immunosuppressive effects on NK cells mediated, at least partly, by secreted factors. In particular, IDO (indoleamine-pyrrole 2,3-dioxygenase) and PGE2 (prostaglandin E2), secreted by MSCs, inhibited proliferation of NK cells and suppressed their cytotoxic effects. PGE2 has also been shown to increase apoptosis of NK cells. Although we did not examine molecular mechanisms in our study, we found that MSC-induced NK cell suppression is caused by soluble factors, not requiring cell–cell contact (Figure 3G).

Importantly, MSCs administered intravenously 24 hours post-MI significantly reduced cardiac neutrophils 7 days post-AMI. The role of NK cells and neutrophils as mediators of inflammatory-induced cardiac functional deterioration has been described. It also has been demonstrated that neutrophils and NK cells are tightly linked so that either cell type can activate or regulate the other based on pathophysiological conditions. Taking together the effect of MSCs on NK cells and neutrophils, we suggest that a major mechanism by which MSCs exert their beneficial myocardial functional effects is by suppressing both NK cells and neutrophils (and thereby the proinflammatory interplay between these cells).
Figure 4. Treatment with mesenchymal stem cells (MSCs) in cardiomyopathy (CM) suppresses neutrophils (Ly6C\textsuperscript{int}Gr1\textsuperscript{hi}). In the ischemic cardiomyopathy model (4 wk post-MI), mice received either intravenous MSC treatment or saline at baseline and week 3 (n=16 per group). At week 6, flow cytometry of the heart demonstrated viable human MSCs in the MSC treatment group (A). Spleen cells were analyzed by fluorescence-activated cell sorting (B) to access the ratio of neutrophils (Ly6C\textsuperscript{int}Gr1\textsuperscript{hi}; C) and monocytes (Continued).
To determine whether there was a causal relation between the MSC-induced decrease in NK cells and the beneficial effects of MSCs post-AMI, we performed NK1.1 antibody-mediated NK cell depletion. NK cell depletion before AMI onset decreased infarct size (Figure 5A). It also significantly changed myocardial functional outcomes by day 20; NK depletion prevented LV adverse remodeling and improved LVEF (Figure 5B through 5D). Yan et al.46 also examined the effect of NK cell depletion on LV function post-MI and reported no improvement in function. These investigators, however, limited their study to 7 days, a time point at which we also showed unaltered LV function.

Furthermore, we examined potential inflammation-related mechanisms responsible for the functional effects of NK cell depletion. We found that at 24 hours post-MI, NK depletion significantly reduced the levels of neutrophils both in the spleen and in the heart (Figure 6A, 6B, 6D, and 6E). These results suggest that the immediate response of neutrophils to AMI is tightly linked to and regulated by NK cells. As neutrophil levels normally decrease later in the course of AMI, as expected, we no longer found a significant difference in splenic or cardiac neutrophil levels at 7 days post-MI despite NK depletion. Interestingly, NK cell depletion significantly increased the proportion of Ly6C\textsuperscript{hi}Gr1\textsuperscript{−} monocytes at both 24 hours and 7 days post-MI in the heart (Figure 6F), raising a question about the role of these cells after AMI in the setting of reduced neutrophil infiltration.\textsuperscript{38,47,48} It would therefore seem that NK cell depletion before AMI leads to an anti-inflammatory effect early during AMI. Furthermore, the persistent NK depletion existing during the later healing phase of the AMI may further contribute to the improved myocardial function we found in the NK cell-depleted mice.

**Table. Comparison of Splenic Cell Populations in the Ischemic Cardiomyopathy Model in MSC-Treated and Control Mice**

<table>
<thead>
<tr>
<th>Splenic Cell Populations</th>
<th>No MSCs</th>
<th>MSCs</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly6G\textsuperscript{−}Ly6C\textsuperscript{−} neutrophils, %</td>
<td>4.9±1.3</td>
<td>2.6±0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ly6G\textsuperscript{−}Ly6C\textsuperscript{−} monocytes, %</td>
<td>9.7±2.8</td>
<td>10.2±2.5</td>
<td>0.67</td>
</tr>
<tr>
<td>F4/80\textsuperscript{−}Ly6C\textsuperscript{−} macrophages, %</td>
<td>4.2±2.8</td>
<td>3.7±2.9</td>
<td>0.68</td>
</tr>
<tr>
<td>NKp46\textsuperscript{+}DX5\textsuperscript{−} natural killer cells, %</td>
<td>3.6±1.6</td>
<td>2.9±1.1</td>
<td>0.13</td>
</tr>
<tr>
<td>DX5\textsuperscript{−}CD3\textsuperscript{−} natural killer T lymphocytes, %</td>
<td>1.6±0.5</td>
<td>1.8±0.7</td>
<td>0.58</td>
</tr>
<tr>
<td>CD3\textsuperscript{+} T lymphocytes, %</td>
<td>31.7±6.8</td>
<td>36.2±7.8</td>
<td>0.09</td>
</tr>
<tr>
<td>CD4\textsuperscript{+} T lymphocytes, %</td>
<td>20.4±5.5</td>
<td>23.8±5.9</td>
<td>0.11</td>
</tr>
<tr>
<td>CD8\textsuperscript{+} T lymphocytes, %</td>
<td>9.6±2.6</td>
<td>10.6±2.2</td>
<td>0.23</td>
</tr>
<tr>
<td>CD19\textsuperscript{−} B lymphocytes, %</td>
<td>39.3±8.6</td>
<td>42.6±7.4</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Flow cytometry of splenocytes enabled comparison of the different immune cell populations between the MSC treatment group and the PBS control group. Populations are presented as a percentage of the total live splenocytes. Data are presented as mean±SD.

**Effects of IV MSC Administration in Mice With Ischemic Cardiomyopathy**

Given the impressive effects we found MSCs produce when administered in the setting of AMI, we decided to examine, in a randomized study, the same hypotheses we examined in the AMI study that (1) intravenous administration of MSCs in murine ischemic cardiomyopathy improves adverse LV remodeling and LV function and (2) one of the major mechanisms leading to such improvement is through a modulation of immune/inflammatory pathways. Our results confirmed the first hypothesis, and although we did not examine the second hypothesis as rigorously as we did in the AMI model, our results were compatible with the validity of the second.

Mice that underwent acute MI (45 minutes of ischemia followed by reperfusion) demonstrated clear echocardiographic evidence of cardiomyopathy after 4 weeks, exhibiting depressed LVEF along with elevated LVEDV and LVESV (Figure 2I through 2K). MSCs were administered intravenously at 4 and at 7 weeks post-AMI.

MSC administration significantly increased LVEF by 27% and decreased LVESV by 25%. These changes were associated with increased LV wall thickness in systole and reduced LVEDV. Meanwhile, the PBS control group experienced progressive adverse remodeling with a 3% reduction in LVEF and a 9% increase in LVESV. MSC-induced improvements became apparent as early as 1 to 3 weeks and, importantly, persisted after the second administration of MSCs. There was also a trend toward stabilization of LVEDV, with the MSC treatment group demonstrating a 7% reduction in LVEDV, whereas the PBS control group had an 8% increase in LVEDV during the 6-week period.

Although fluorescent imaging (Figure 1G) and flow cytometry demonstrated that the intravenously administered MSCs homed to the myocardium in these mice with ischemic cardiomyopathy and remained viable (Figure 4A), only a small percentage of the intravenously injected MSCs engrafted in the myocardium. Despite this, and similar to our studies in acute MI, intravenously administered MSCs exerted systemic immunomodulatory effects marked by a highly significant decrease in splenic Ly6C\textsuperscript{hi}/Ly6G\textsuperscript{−} neutrophils (Figure 4B and 4C). Although these proinflammatory cells have been associated with adverse remodeling, they have a complex role in MI and progression to heart failure.\textsuperscript{49} Recent studies demonstrate that neutrophils play an integral role in switching macrophages to a reparative phenotype.\textsuperscript{47} This complex interplay between resident macrophages and the innate immune response after MI\textsuperscript{50–53} requires further study, especially given the impact MSCs play in modulating the inflammatory response after both acute MI and in chronic ischemic cardiomyopathy.

MSCs also alter the balance existing between peripheral and resident cardiac myeloid cell populations that orchestrate repair after MI and modulate LV remodeling.\textsuperscript{54} As demonstrated in Online Figure IV, MSC treatment significantly modulates...
Figure 5. In vivo natural killer (NK) cell depletion improves cardiac function post-myocardial infarction (MI). Antibody against NK1.1 was injected intravenously 24 h before MI induction (n=12 per group). Echocardiography was performed on days 7 and 20 post-MI. At 21 d post-MI, heart sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and analyzed for infarct size, calculated as percent of left ventricle (LV; A). Echocardiographic analysis included assessment of LV ejection fraction (B), LV end-diastolic volume (C), and LV end-systolic volume (D). Fluorescence-activated cell sorting was performed on the spleen 21 d post-MI (n=12 per group), confirming NK depletion (E and F) and assessed the composition of neutrophils and monocytes (G).
the T-lymphocyte populations, affecting the inflammatory milieu and leading to a cardioprotective phenotype with reduced neutrophil:lymphocyte ratio, a finding supported in the clinical literature. Our study therefore supports the concept that MSC-mediated systemic immunomodulatory effects play a role in the highly significant MSC-induced improvement in LV function and in adverse LV remodeling in mice with ischemic cardiomyopathy. Despite only a trend for reduction

Figure 6. Natural killer (NK) cell depletion at the time of myocardial infarction (MI) in mice effects neutrophils (Ly6C<sup>hi</sup>Gr1<sup>hi</sup>) and monocytes (Ly6C<sup>hi</sup>Gr1<sup>-</sup>) in the spleen and the heart. Anti-NK cell (NK1.1) antibody was administered intravenously immediately after MI. The effect of NK depletion on the ratio of neutrophils (Ly6C<sup>hi</sup>Gr1<sup>hi</sup>) and monocytes (Ly6C<sup>hi</sup>Gr1<sup>-</sup>) was analyzed by fluorescence-activated cell sorting, both at 24 h and at 7 days post-MI, in the spleen (A–C) and in the heart (D–F; n=8 in each group).
in splenic NK cells with human MSCs in our ischemic cardiomyopathy model, a recent phase IIa randomized clinical trial in nonischemic cardiomyopathy using these same human MSCs demonstrated a significant reduction in circulating NK cells. Interestingly, the degree of reduction significantly correlated with improvement in LVEF.60

Our findings contribute to the expanding body of data suggesting that the spleen plays an important role in mobilizing the immune/inflammatory response to acute and chronic myocardial injury. The existence of a cardiосplenic axis was first suggested by Swirski et al., who demonstrated rapid depletion of splenic monocytes shortly after acute MI, a change accompanied by a massive influx of Ly-6C<sup>+</sup> monocytes into ischemic myocardium; this influx was impaired by splenectomy. Ismail et al demonstrated an increase in splenic size in a murine model of chronic ischemic heart failure accompanied by a marked depletion of splenic proinflammatory monocytes and an increased cardiac monocyte population. Interestingly, splenectomy not only decreased cardiac macrophages and dendritic cells but also attenuated the adverse LV remodeling. This supports an integral role for the spleen in chronic heart failure. Although <sup>18</sup>F-fluorodeoxyglucose positron emission tomographic imaging has validated the cardiосplenic axis in patients with acute MI, we are unaware of any published studies examining the cardiосplenic axis in patients with chronic heart failure.

Previous clinical trials targeting inflammation in heart failure have failed to show clinical benefit.55,56 However, these trials focused on inhibition of inflammation largely through a single mechanism. MSCs secrete numerous growth factors and cytokines influencing a diverse array of pathways, such as those related to multiple inflammatory pathways, adverse LV remodeling, angiogenesis, tissue healing, apoptosis, mitochondrial dysfunction, microvascular dysfunction, and collagen deposition.9–14 Such multifunctional activities provide a rationale for why MSCs might provide a more effective therapeutic strategy than drugs with a more limited range of activities.

**Issues Relating to the Xenograft Model and to the Immune Status of MSCs**

A critical question that must be addressed about the validity of our results is whether the xenograft model we used, in which human MSCs are injected into mice, provides results that would be expected to be seen when allogenic human MSCs are injected into humans. The literature on this subject is extensive and is discussed in detail in the Online Discussion.

The bottom line is that MSCs are not immune privileged, as originally proposed. They eventually are eliminated from mismatched hosts through immune responses. This probably explains, in part, the fact that fewer human versus murine MSCs engrafted in the myocardium and spleen 24 hours after injection in mice with AMI (Online Figure II). However, MSCs do persist for a limited time (longer than other types of mismatched cells) and are, thus, immune evasive but not immune privileged.30,32 Therefore, in either xenograft models of disease or in the setting of administering allogenic MSCs to patients with cardiac (or other) diseases, the issue is whether MSCs persist long enough to exert a therapeutic effect. The literature supports a positive response to this question.60–63 Most relevantly, our data indicate that viable human MSCs persist for at least 20 days when administered IV into mice and that this duration of persistence is sufficient to lead to significant immunomodulatory effects and to marked improvement in LV function.

**Conclusion**

In an occlusion/reperfusion model of AMI, progressive adverse LV remodeling and deterioration in LV function occurs during the first few weeks post-AMI in mice with large infarcts. Importantly, intravenously administered MSCs prevent these deleterious effects, with a major contributory mechanism being an MSC-induced modulation of immune/inflammatory responses. This effect includes a suppressive effect on NK cells, which we show decreases in both splenic and cardiac neutrophils and improves adverse remodeling and LV function. These results are compatible with the concept that excessive inflammation is an important mechanism contributing to progressive LV dysfunction post-AMI, and that at least one of the mechanisms responsible for the beneficial myocardial effects of intravenously administered MSCs is through systemically mediated anti-inflammatory activities. Intravenously administered MSCs also significantly improved LV function in mice with ischemic cardiomyopathy, changes also accompanied by MSC-induced immunomodulatory effects. Our results therefore raise the possibility that intravenously administered MSCs may be a practical and effective therapeutic strategy for the treatment of patients with large infarcts and patients with ischemic cardiomyopathy. The validity of these concepts will have to await the results of appropriate clinical trials.

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**Disclosures**

S.E. Epstein is a consultant to and holds equity interest in CardioCell. S. Sikora, A. Kharazi, and G. Vertelov are employees of CardioCell, LLC.

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Intravenously Delivered Mesenchymal Stem Cells: Systemic Anti-Inflammatory Effects Improve Left Ventricular Dysfunction in Acute Myocardial Infarction and Ischemic Cardiomyopathy

Dror Luger, Michael J. Lipinski, Peter C. Westman, David K. Glover, Julien Dimastromatteo, Juan C. Frias, M. Teresa Albelda, Sergey Sikora, Alex Kharazi, Grigory Vertelov, Ron Waksman and Stephen E. Epstein

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Material and Methods

Acute myocardial infarction model:

CD1 male mice (8-10 weeks of age) were subjected to operative intervention to create acute MI. Mice were anesthetized with sodium pentobarbital 70 mh/kg administered by intaperitoneal injection and hair was removed from the left axilla. After anesthesia, mice were intubated and ventilated with room air using positive pressure respirator model 845 (Minivent, Harvard apparatus). Left thoracotomy was performed at the fourth inter-costal space to expose the heart. Self-retaining microretractors were used to separate the 3rd and 4th ribs enough to adequately expose the heart, while leaving the ribs intact. After opening the pericardium, the left anterior descending coronary artery (LAD) will be temporarily ligated with a 7-0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. For the ischemia reperfusion (IR) model, a 2-mm section of PE-10 tubing will be placed over the LAD and the 7-0 silk suture will be tied into a slipknot on top of the tubing to occlude the LAD for 45 min (inducing ischemia). Ligation will be deemed successful when the anterior wall of the left ventricle turns pale. The chest wall was approximated and covered with a piece of moistened gauze to prevent desiccation during ischemia. Reperfusion was achieved by removal of the PE-10 tubing. This allowed release of the occlusion and reperfusion of the formerly ischemic myocardium. Cessation of positive pressure ventilation enabled spontaneous inspiratory effort to fully inflate the lungs immediately prior to closing the chest wall. The thoracotomy site was closed in layers using 5-0 prolene sutures for closing the rib cage and then the skin. Animals were kept on a heating pad until they recover after with they were housed individually in cages.

Human Mesenchymal Stem Cells

Primary frozen bone marrow derived human MSCs were received from Stemeda (San Diego, Ca.) and cultured as described below. After expansion, cells were aliquoted and frozen for future study. Culture conditions included: media (DMEM/F12-GlutaMax (Invitrogen), HEPES Buffer 1X (Invitrogen), non-essential amino acids 1X (Invitrogen), sodium pyruvate 1X (Invitrogen), fetal bovine serum 10% (Gibco), antibiotic-antimycotic 1X (Gibco), 2 mercaptho-ethanol 1ul/ml (Sigma), fibroblast growth factor (bFGF) 20 ng/ml (Shenandoah Biotechnology). Cells were cultured in 15 cm dishes (Falcon). Cell confluence was achieved at 8-12 days, then cells were passed to new dishes at 1X10⁶ cells/dish. Cells from passage 3-6 were used for experiments. Unless otherwise specified, 2 x106 MSCs in 0.2 ml were injected in each mouse into the lateral tail vein 24 hours following AMI. In vitro experiments were performed utilizing these cells incubated with murine serum and the degree of complement-mediated cell lysis was assessed by determining cell viability.

Radiolabeling of MSCs

Molar excess of oxyquinoline (oxine) was incubated with indium-111 HCl (PerkinElmer) for 30 minutes at room temperature in HEPES Buffer with 10% dimethyl sulfoxide, enabling formation of indium-111 oxine. Indium-111 has a half-life of 2.8 days with photon energies of 171 and 245 keV. MSCs were resuspended with indium-111
oxine solution and allowed to incubate at room temperature for one hour. Cells were washed three times with normal saline after which percent cellular viability and MSC counting was then determined by counting cells following Trypan Blue staining. Tail vein injection of 1.0 x 10^8 radiolabeled MSCs was then performed 24 hours following AMI (n=10) and in control mice without MI (n=10). The μCi of indium-111 per injected dose was determined by measuring the radioactivity of the syringe before and following injection into the mouse using a Radioisotope Dose Calibrator (Searle Radiographics).

Ex Vivo Nuclear Imaging of MSCs

Approximately 20 hours following intravenous injection of radiolabeled MSCs, the recipient mice were euthanized by intraperitoneal injection of sodium pentobarbital overdose, the vasculature was perfused with PBS, and the organs were harvested. Short-axis cross-sections of the hearts were exposed to a high sensitivity, medium resolution phosphor imaging screen (PerkinElmer). After 20 hours, the phosphor imaging screen was scanned using a PerkinElmer Cyclone Plus Phosphor Imaging System. Signal intensity analysis for each heart was performed in a blinded fashion using Sante DICOM software and was adjusted for injected radioactive dose per animal and background using the formula: (Mean Signal Intensity of heart - Background Signal Intensity) / Injected Radioactive Dose in μCi). Signal intensity of the LAD region was compared with the posterior wall to assess difference in signal intensity within the infarct region. Comparison of MSC content based on indium-111 was then determined for different organs using a Minaxi 5500 Gamma Counter using an indium-111 window setting (160 to 485 keV). Gamma counting for each sample occurred over a five minute period to generate an average number of counts per minute (CPM) and the adjusted gamma counts for each organ was determined by the formula: ((CPM of tissue - Background CPM) / Weight of the tissue in grams / Injected radioactive dose in μCi).

Ex-Vivo fluorescent Imaging

MSCs were fluorescently labeled by in vitro incubation for 30 minutes with Qdot 705 (Invitrogen) and injected as above. To identify tissue engrafted MSCs, mice were sacrificed and organs were harvested; fluorescent optical imaging was performed with an in-vivo imaging system (IVIS) 100 (Perkin Elmer, USA) at the relevant channel.

Echocardiograms

Echocardiograms were performed with a 30 mHz ultrasound probe (VEVO 1100 Imaging system, VisualSonics Inc) under light anesthesia with sodium pentobarbital (30-40 mg/kg intraperitoneal injection). Short axis 2-dimensional echocardiogram images were acquired at the midpapillary level of the LV and stored as digital loops. Anterior wall thickness at end-systole (AWTs), LV end-systolic diameter, posterior wall thickness at end-systole (PWTs), anterior wall thickness at end-diastole (AWTd), LV end-diastolic diameter, and posterior wall thickness at end-diastole (PWTd) were calculated by manual tracings of the endocardial border. These values were then utilized to calculate LV ejection fraction (LVEF), end-systolic volume (LVESV), and end-diastolic volume (LVEDV). Echocardiography was obtained at baseline prior to AMI surgery then at day 3, 7 and 20 post MI.

Preparation of single cell suspensions for flow cytometry
Single cell suspensions for flow cytometry and FACS were prepared in FACS buffer. Mice were perfused with PBS through the LV to clear the blood system. Spleens were mashed with the plunger of a 3ml syringe through a 70µm cell strainer (BD Falcon). Heart tissue had been cut into 1mm pieces that were digested for 30 minutes with collagenase II and DNAse. Single cell suspension was collected by mashing the digested tissue through a 70µm cell strainer. Blood was obtained by cardiac puncture. Blood samples were lysed twice in ACK buffer for leukocytes isolation. Cells were recovered in complete medium, filtered through 40µm cell strainers, counted and maintained on ice for further analysis.

Flow Cytometry

Cells surface Fc receptors were blocked by incubation with anti-Fc (CD32/CD16, clone 93) (BioLegend, CA) for 15min. Cells were stained with the relevant antibodies in FACS buffer for 30min at 4°C, washed and stained with 7-AAD to enable exclusion of dead cells. Anti-mouse antibodies used for FACS staining (BioLegend, CA) were: CD19, B220, CD5, CD3, CD4, CD8, CD11b, Ly6C, Ly6G, NKp46, DX5, NKG2D, CCR2, CX3CR. Flow cytometry reading was done on FACS Calipur (BD CA.). Flow cytometry analysis was performed with FlowJo (Ashland, OR).

TTC staining

Following the final echocardiogram, mice were administered a lethal dose of sodium pentobarbital. Whole blood was harvested from mice at the time of sacrifice by right ventricular puncture and the vasculature was perfused by LV puncture using PBS. The heart was quickly excised, short-axis or axial cross-sections of the heart were cut to 1.0 mm thickness (Zivic Instruments, Pittsburgh, PA), and incubated at 37°C in 1% TTC for 10 minutes. Heart sections were then fixed in 2% paraformaldehyde overnight. Each stained cardiac section was photographed and infarcted area was analyzed using Image J software (NIH, Bethesda, MD). The areas were defined as follows: the infarct area consists of the TTC-negative staining region (white/tan), and the non-ischemic region consists of the TTC positively staining regions (red). Myocardial infarct size was calculated as a percentage of the total left ventricle area from the slices distal to the ligature.

In-vivo NK cells depletion

For in-vivo NK cells depletion the antibody anti-NK1.1 clone was injected intravenously (100mg/ mouse) 24h prior to MI surgery. The experiment included a control group (MI, n=12) and NK depletion group (MI + anti NK1.1, n=12). Echocardiograms were measured at 7 and 21 days post MI. At 21 days post MI hearts were analyzed by TTC staining for infarct size and the ratio of spleen immune cells was analyzed by FACS. A number of mice were dedicated to validate in-vivo NK cells depletion on day 7 post injection. NK depletion was evaluated by FACS analysis on splenocytes.

Statistical Analysis

Continuous variables are presented as mean ± standard deviation unless otherwise specified. Comparison of variables between the two groups was performed using unpaired student T-test or Mann-Whitney U or Wilcoxon Rank-Sum Test in the
case of non-parametric data with non-normal distribution. Aspin-Welch test was utilized in the case of unequal variance of the means. Correlation analysis was performed with linear regression to provide a goodness of fit line and Pearson correlation coefficient. Statistical analysis was performed with Number Crunching System 2007 software (Kaysville, UT) and Graphpad Prism 6.0 software (San Diego, CA). A test was considered statistically significant with p-value less than 0.05.

Online Discussion

Xenograft Model

When designing the current investigation, we made two important decisions relating to the model. First, we decided to use human MSCs. The reason being that mouse MSCs have many very different characteristics from human-derived MSCs. We were therefore concerned that the results we would have obtained with mouse MSCs might not accurately reflect effects of human MSCs in human trials. This decision to use human MSCs posed the dilemma of whether we had to use immunocompromised mice to avoid the immune responses inherent in xenograft models. Use of immunocompromised mice, however, would seriously limit the relevance of the results of our studies, based as they are on the hypotheses that 1) the progressive LV dysfunction observed in our mouse models of AMI and of ischemic cardiomyopathy was caused, at least in part, by excessive immune/inflammatory activity, and 2) one of the important mechanisms leading to possible beneficial effects of MSCs would derive from their immunomodulatory effects.

However, use of a xenograft model—at least when MSCs are being use across species—may in fact be possible. Jiang and colleagues conducted an extensive literature search and identified 13 studies in which human MSCs were injected into immunocompetent mice or rats. Each of these studies demonstrated by the time of study termination (5 days to 6 weeks), depending on endpoints evaluated, either that MSCs continued to be viable, or that the cells had contributed to positive outcomes. None of these studies demonstrated evidence of rejection.

These results were interpreted as being consistent with the concept that MSCs are immune privileged. However, a more nuanced perspective of the immune "privileged" status MSCs has more recently evolved. In their review, these authors pointed out that administration of allogenic MSCs, or MSCs administered cross-species (human MSC administered to mice), do not persist indefinitely; they postulated it is likely that an active immunological process is responsible for this limited persistence. Despite this lack of persistence, indicating that MSCs are not in reality immune privileged, Ankrum and colleagues discussed the relative immune evasive capacity of MSCs. They presented the results of a study in which the relative persistence of allo-fibroblasts vs. allo-MSCs was compared. The study showed that fibroblasts died by day 10 and mMSCs by day 20—in other words, although the MSCs were not immune privileged, they had some protection against immune rejection (the authors refer to this as immune evasive) resulting in longer persistence than the non-MSCs. This concept was also documented by using murine MSCs transfected with the gene expressing erythropoietin as a reporter for persistent MSC functionality. MSCs were injected subcutaneously in either major histocompatibility complex (MHC)-mismatched allogeneic or matched syngeneic mice. Although expression of erythropoietin lasted for a longer
time in the syngeneic mice, the mismatched MSCs still produced erythropoietin for over 30 days.

These results suggest that although MSCs are not immune privileged and eventually are eliminated from mismatched hosts through immune responses, they still could exert therapeutic actions if the relevant activities of the MSCs need only a limited time to produce their effects. Such a mechanism has been referred to as a “hit-and-vanish” or “hit and run” mechanism\(^1\). Therefore, the question in either xenograft models of disease or in the setting of administering allogenic MSCs to patients with cardiac (or other) diseases becomes not one of “do MSCs have a total immune privileged status” but, given their immune evasive capacity, “do they persist long enough to exert a therapeutic effect”. Our data indicate that human MSCs persist for at least 20 days when administered IV into mice, and that this duration of persistence is sufficient to lead to marked improvement in LV function.

Online Figure Legends

**Online Figure I: Schematic for the experimental protocol**

Acute MI Timeline.
Thirty-two male CD1 mice were randomized based on body weight and underwent baseline echocardiograms (Blue arrow). Thereafter, mice underwent acute myocardial infarction (AMI) surgery with 45 minutes of ischemia followed by reperfusion. At 24h following MI treatment group were IV injected with MSCs (2x10\(^6\)/mouse) (green arrow) and a control group were IV injected with the same volume of PBS (red arrow). Follow up echocardiography were done on days 3, 7 and 20. At 21 days hearts were harvested and analyzed for infarct size. Spleen cells were analyzed for the ratio of the different immune cells. Echocardiograph measurements were further analyzed.

Ischemic Cardiomyopathy Timeline.
Thirty-two male CD1 mice underwent acute myocardial infarction (AMI) surgery with 45 minutes of ischemia followed by reperfusion. After 4 weeks the mice underwent baseline echocardiography (blue arrows) with representative M-mode echocardiograms seen at the top and bottom left. The top left echocardiogram demonstrates how measurements were obtained for anterior and posterior wall thickness in systole (AWTs and PWTs), anterior and posterior wall thickness in diastole (AWTd and PWTd), left ventricular end-systolic diameter (LVESD), and left ventricular end-diastolic diameter (LVEDD). The representative baseline M-mode echocardiograms demonstrate evidence of prior MI with reduced AWTs, increased LVESD and LVEDD, and decreased left ventricular ejection fraction (LVEF). Mice were then randomized based on LVEF into two equal groups and received either intravenous tail vein injection of 2.0 x 10\(^6\) MSCs grown under hypoxic conditions (green arrow) or PBS control (red arrow). The mice then underwent repeat echocardiography at weeks 1 and 3 (blue arrows). The mice then underwent repeat intravenous injection of MSCs (green arrow) or PBS control (red arrow). The mice then underwent repeat echocardiography at weeks 4 and 6 (blue arrows), after which the animals were euthanized and their organs were collected for analysis. Paired representative M-mode echocardiograms for a mouse in the MSC treatment group (top right) demonstrated improved systolic wall thickening with reduced LVESD and increased LVEF while there was no improvement in the representative M-mode echocardiogram at week 6 for the PBS control mouse.

**Online Figure II: In vivo bio-distribution of murine MSCs in AMI**
Radiolabeled murine MSCs grown under chronic hypoxia (5% O2) were injected intravenously in mice 24h post-MI or in mice that underwent sham surgery, (n=10/group). MSC content in different organs was measured 24h post-injection using a gamma well counter. Heart and spleen MSC engraftment was calculated per gram tissue (top panel). In the bottom panel, we provide comparison of trafficking of human and murine MSCs to the heart and spleen 24h post injection using the gamma well counter in mice that underwent AMI surgery 24 hours prior to injection.

**Online Figure III: Intravenously-administered human MSCs improve cardiac function in murine acute MI and ischemic cardiomyopathy models.**

MSCs were administered 24h post-MI and their therapeutic effect was evaluated. Echocardiography was performed at baseline prior to MI and then again at days 3, 7, and 20 post-MI. Comparison is provided for anterior wall thickness in diastole (A), posterior wall thickness in diastole (B), anterior wall thickness in systole (C), and posterior wall thickness in systole (D) for the MSC treatment vs. control groups (n=16 per group). In the ischemic cardiomyopathy model (4 weeks post-MI), mice received either intravenous MSC treatment or saline at baseline and week 3 (n=16 per group). Serial echocardiography was performed at baseline, week 1, week 3, week 4, and week 6. Comparison is provided for anterior wall thickness in diastole (E), posterior wall thickness in diastole (F), anterior wall thickness in systole (G), and posterior wall thickness in systole (H) for the MSC treatment vs. control groups in the ischemic cardiomyopathy model. *p<0.05, **p<0.01 in comparison to baseline.

**Online Figure IV: Correlation of T lymphocyte populations with LV dimensions and function**

Flow cytometry analysis determined the T lymphocyte population (CD3+) and subpopulations (CD4+ or CD8+), which were correlated with echocardiographic parameters. Correlation matrices are presented plotting the splenic CD3+ cells as a percentage of the total live splenocytes (A, B, and C), the splenic CD4+ cells as a percentage of the total live splenocytes (D, E, and F), and the splenic CD8+ cells as a percentage of the total live splenocytes (G, H, and I) on the X-axis. The Y-axis plots LVEDV at 6 weeks (A, D, G), LVESV at 6 weeks (B, E, H), and LVEF at 6 weeks (C, F, I). Data from the MSC treatment group (green circles) and from the PBS control group (red squares) is plotted for each animal. Correlation analysis with best fit lines are presented for each group with corresponding R^2 value and p value. Among MSC treatment mice, there was a correlation between higher T lymphocyte populations with improved LV volumes and LV function (with the exception of G) while there was no correlation among mice in the PBS control group.

**References**


Figure II

Murine MSC Distribution Study Supplement

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Comparison of Human and Murine MSC Distribution

- Human MSCs
- Murine MSCs

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Figure III

**Acute Myocardial Infarction**

A

![Graph A: Anterior Wall Thickness](image)

B

![Graph B: Posterior Wall Thickness in Diastole](image)

**Ischemic Cardiomyopathy**

C

![Graph C: Anterior Wall Thickness](image)

D

![Graph D: Posterior Wall Thickness in Systole](image)

E

![Graph E: Anterior Wall Thickness in Diastole](image)

F

![Graph F: Posterior Wall Thickness in Systole](image)

G

![Graph G: Anterior Wall Thickness in Systole](image)

H

![Graph H: Posterior Wall Thickness in Diastole](image)
Figure IV