Left Ventricular Dysfunction Switches Mesenchymal Stromal Cells Toward an Inflammatory Phenotype and Impairs Their Reparative Properties via Toll-like Receptor-4

Running Title: Naftali-Shani et al.; Inflammatory Mesenchymal Stromal Cells

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Abstract

Background—Little is known about the potentially unfavorable effects of mesenchymal stromal cell (MSC) activation on the heart. MSCs can respond to tissue injury by anti or pro-inflammatory activation. We sought to study the potential negative interaction between left ventricular dysfunction (LVD) and MSC activation.

Methods—We isolated MSCs from cardiac (c) and subcutaneous (sc) fat tissues of mice with LVD, 28 days after myocardial infarction (MI), or sham operation. To evaluate the effect of LVD on MSCs, we characterized cMSCs and scMSCs in vitro. Subsequently, we injected MSCs or saline into the infarcted myocardium of mice and evaluated left ventricular (LV) remodeling, and function, 28 days after MI. To test the hypothesis that toll-like receptor 4 (TLR4) mediates pro-inflammatory polarization of MSCs, we characterized cMSCs from TLR4-deficient and wild-type (WT) mice after inflammatory stimulation in vitro. Next, we transplanted cMSCs from TLR4-/- and WT male mice into the infarcted myocardium of female WT mice and evaluated infarct size, MSC retention, inflammation, remodeling and function, after seven days.

Results—LVD switched cMSCs toward an inflammatory phenotype, with increased secretion of inflammatory cytokines, as well as chemokines. The effect of LVD on scMSCs was less remarkable. While transplantation of cMSCs and scMSCs from LVD and sham hearts did not improve LV remodeling and function, cMSCs from LVD exacerbated anterior wall thinning, 28 days after MI. The inflammatory polarization of cMSCs by LVD was mediated by TLR4, as we found less secretion of inflammatory cytokines and higher secretion of anti-inflammatory cytokines from activated cMSCs of TLR4-deficient mice, compared with WT cMSCs. Significantly, TLR4-deficiency preserved the expression of CD47 (“don’t eat me” signal) on cMSCs after both TLR4 stimulation in vitro and transplantation into the infarcted heart. Compared with WT cMSCs and saline, TLR4-/- cMSCs survived in the cardiac tissue and maintained their reparative properties, reduced infarct size, increased scar thickness and attenuated LV dilatation, seven days after MI.

Conclusions The environment of the failing and infarcted myocardium drives resident and transplanted MSCs toward a pro-inflammatory phenotype, and restricts their survival and reparative effects in a mechanism mediated by TLR4.

Key-Words: heart failure; inflammation; mesenchymal stem cell; Toll-like receptor 4
Clinical Perspective

What is new?

- Resident and implanted mesenchymal stromal/stem cells (MSCs) respond to myocardial injury, inflammation, and left ventricular (LV) dysfunction by pro-inflammatory activation.
- Pro-inflammatory MSCs contribute to adverse LV remodeling and dysfunction.
- Toll-like receptor-4 (TLR4) deficiency in MSCs attenuates their pro-inflammatory activation, improves their reparative properties, graft survival, infarct repair, and LV remodeling.

What are the clinical implications?

- Targeting TLR4 in MSCs could improve the safety and efficacy of cell therapy.
- Our findings could guide the selection of MSC source when deciding between autologous vs. donor MSCs for cell-based therapy in heart failure.
Introduction

Mesenchymal stromal cells (MSCs), also known as mesenchymal stem cells, are resident in almost all tissues, including the heart, and play a major role in tissue repair and regeneration.1-3 MSCs possess immunomodulatory, anti-inflammatory properties4, 5 and have emerged as a viable source for cardiac cell therapy. The favorable effects of MSCs on the injured myocardium have been extensively studied, and are attributed to secretion of immunomodulatory, trophic factors, and chemokines.6 Because of their cardiovascular-associated features,7, 8 cardiac MSCs (cMSCs) may represent a more suitable cell source for cardiac repair. Little is known, however, about the effect of the injured and fibrotic myocardium on the function of both resident and transplanted MSCs, and the potential adverse effects of the transplanted cells. MSCs can act as a sensor of myocardial injury and inflammation and respond by anti-inflammatory or pro-inflammatory activation4, 9, 10 Subject to the types of inflammatory responses (acute or chronic), MSCs may either suppress inflammation and enhance repair of the injured tissue, or preserve a chronic inflammatory response, leading to fibrosis and adverse tissue remodeling.4, 11 This is important because left ventricular dysfunction (LVD) and heart failure are associated with low-grade, local and systemic inflammation,12 able to modulate the function of resident and transplanted MSCs.

In view of the possible negative interactions between inflammation and MSCs, there is a need to determine not only whether, but also how LVD affects the function of MSCs. Understanding the influence of LVD on the function of resident and transplanted MSCs is essential to improve the outcome of cell-based therapy. In the present study, we aimed to determine the effect of chronic LVD on the phenotype and function of resident cardiac and peripheral MSCs, and to elucidate how the activation of MSCs affects the development of LVD.
We isolated MSCs from mice with chronic LVD after extensive myocardial infarction (MI). To test the hypothesis that Toll-like receptor 4 (TLR4) mediates the effects of LVD on MSCs, we used cMSCs from TLR4-deficient mice. Our results could help to improve the safety and efficacy of MSC-based therapy in cardiovascular regenerative medicine.

**Methods**

All animal experiments were complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA), and were approved by the Sheba Medical Center Institutional Animal Care and Use Committee. To isolate and characterize MSCs from LVD or sham mice we used 11-week old, 22gr, Balb/C female mice (Harlan, Jerusalem, Israel). To induce MI for MSC transplantation experiments, we used 11-week old, 22gr, Balb/C female mice (Harlan, Jerusalem, Israel). To determine the role of TLR4 in cMSCs we used 9-week old, transgenic TLR4−/− (C57BL/10ScN) male mice.13 As a control for the TLR4−/− mice, and for the isolation of spleen macrophages (MΦs), we used 9-week old, C57BL/6 male mice (Harlan, Jerusalem, Israel).

**Model of MI, Sham MI, and Cell Transplantation**

The model of MI and LVD in mouse has been previously described.3,14,15 In brief, mice (female Balb/C) were anesthetized by inhalation of 2% isoflurane/98% O2. The chest was opened by gentle dissection and left thoracotomy via the fourth intercostal space. The pericardium was removed to provide access to the left ventricular free wall. A 9-0 prolene suture was used to ligate the left anterior descending (LAD) coronary artery at the lower border of the left atrium. MI was confirmed by visual blanching distal to the occlusion site, and by echocardiography at 24h post-MI. For sham MI, the suture was passed around the coronary artery and removed.
without ligation. The thoracic incisions were closed with 5-0 silk sutures. For cell transplantation experiments, we injected the different MSCs (10^5) or saline (20ul) into the ischemic border zone, one minute after artery occlusion. Mice with LV fractional shortening above 40% at day 1 after MI were excluded from the study (n=3). In the MI/sham MSC study, 38 mice were treated with either cMSCs or subcutaneous (sc) fat MSCs, which were obtained from one mouse with MI or sham operation, and followed up for 28 days. Perioperative mortality was 58% (52 out of 90). In the second study (TLR4-deficient MSC transplantation), 21 mice were treated with cMSCs pooled from three TLR4^-/- or WT mice and followed up for 7 days. Perioperative mortality was 25% (7 out of 28). To assess the survival of MSCs, 7 days after transplantation, we injected 10^5 mCherry-labeled MSCs (20ul) from male C57BL/6 mice into the infarcted heart of female Balb/C mice (n=3), immediately after MI.

**Echocardiography**

To assess LV remodeling and function after cell therapy, we performed transthoracic echocardiography with a special small animal echocardiography system (Vevo 2100 Imaging System; VisualSonics, Toronto, Canada) equipped with a 22–55 MHz linear-array transducer (MS550D MicroScan Transducer). Echocardiographic studies were done at day 1 and day 7 or 28 after MI and MSC transplantation. Light anesthesia was induced by inhalation of 2% isoflurane/98% O_2, and subsequently maintained by 0.5-1% isoflurane. We controlled the isoflurane flow to maintain the heart rate at >400 bpm (mean 428±8 bpm). All measurements were averaged for 3 consecutive cardiac cycles, and performed by an experienced technician who was blinded to the treatment groups. Percent of change from first value was calculated for each animal as follows: [(follow-up value – first value)/first value] × 100; Ejection fraction =
[(LV vol d-LV vol s)/LV vol d] x100; Fractional shortening = [(LVDD-LVSD)/LVDD] x100;
Fractional area change = [(LVDA-LVSA)/LVDA] x100.

**Isolation of MSCs**

To evaluate the effect of LVD on resident MSCs, we isolated the cells from the heart and sc fat (inguinal fat pad) of Balb/C female mice, 28 days after extensive MI (n=9) or sham operation (n=7). To determine the role of *TLR4* in cMSC polarization, we isolated cells from the hearts of *TLR4*−/− (n=5) and C57BL/6 (WT) (n=4) male mice.

Cells were extracted with an enzymatic digestion mixture as previously described,3 using three cycles of incubation at 37°C for 10 minutes. Plastic-adhered cells were incubated at 37ºC in humid air with 5% CO2 and grown in DMEM (Biological Industries, Beit Haemek, Israel) with 15% FBS (Biological Industries, Beit Haemek, Israel), 1% penicillin-streptomycin (Biological Industries, Beit Haemek, Israel), 1% L-Glutathione, (Sigma-Aldrich, Rehovot, Israel), 1% MEM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), and 0.1mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA, USA). The medium was changed 3 days after plating and subsequently every 3 or 4 days. In each group of each experiment, we used cultured MSCs at passage 3 (from a minimum of 3 mice).

**Characterization of MSCs**

MSCs were defined according to the three criteria of the International Society for Cellular Therapy 16: (a) Adhesion to plastic (b) expression of a specific combination of surface markers (c) differentiation potential (tri-lineage differentiation into adipocytes, osteoblasts, and chondrocytes).17
Flow Cytometry

To define the phenotype of MSCs, we analyzed cultured cells at passage 3 for common mouse MSC markers by flow cytometry, using the following fluorescence anti-mouse antibodies: CD105-APC, CD90-FITC, CD11b-FITC, CD45-FITC, CD31-FITC, TER119-FITC, mouse IgG2a-APC, IgG2a-PE and IgG2a-FITC (BioLegend, San-Diego, CA, USA).

To determine the effect of TLR4 on CD47 expression in cultured MSCs at passage 3, we analyzed MSCs from TLR4−/− and WT mice by flow cytometry, using anti-mouse antibody against CD47-PE (BioLegend, San-Diego, CA, USA). MSCs were analyzed with or without activation of TLR4 by LPS (10 or 100ng/ml) for 20 hours. Labeled cells (0.5x10⁶) from each sample were acquired and analyzed using FACS Calibur Cytofluorimeter (Cyteck Development, Fremont, CA, USA) with Flowjo Software (Tree Star, Ashland, OR, USA).

Differentiation Assays

To examine the in vitro differentiation potential of the different MSCs into osteogenic, adipogenic, and chondrogenic lineages, we used specifically formulated cell culture media (MesenCult™ Osteogenic Stimulatory Kit (Mouse), MesenCult™ Adipogenic Stimulatory Supplements (Mouse), and MesenCult™-ACF Chondrogenic Differentiation Medium (all from STEMCELL Technologies™ Inc., Vancouver, BC, Canada).

Briefly, for osteogenic and adipogenic differentiation, according to the manufacturer's instructions, MSCs were seeded at a concentration of 2.5x10⁴/cm² in a 24-well plate and grown for 2-3 weeks with osteogenic or adipogenic medium changed twice a week. Osteogenic differentiation was detected by Alizarin red S staining (0.5% in distilled water, pH=4.2) and adipogenesis was detected by Oil red O staining (0.3% in isopropanol) (both from Sigma-Aldrich, Steinheim, Germany). For chondrogenic differentiation, MSCs were grown in a 3D
micro-mass culture system. At a concentration of 0.5x10^6/15 ml in a polypropylene tube, cells were centrifuged at 300g for 5 minutes, then supernatant was removed and cell pellets were treated for 14 days with chondrogenic medium changed twice a week. Chondrogenic differentiation was detected by Alcian blue staining (1% in 0.1N HCl) (Sigma-Aldrich, Steinheim, Germany).

**Proliferation Assay**

To measure MSC growth rate we used a cell proliferation assay. Cells at passage 3 were cultured at 37°C in 96-well plates at a concentration of 3000 cells per well. Then, we measured optical density in triplicate wells for each MSC group using a cell proliferation kit XTT-based colorimetric assay (Biological Industries, Beit Haemek, Israel) for 4-5 consecutive days. Each assay was performed on 3-4 primary cell cultures from each MSC group.

**Cytokine Array**

To define the levels of cytokine secretion from MSCs, we used Q-Plex™ Mouse Cytokine Inflammation (14-plex) array (Quansys Biosciences Multiplex ELISA, West Logan, UT, USA). Briefly, we cultured MSCs for 72 hours and collected their secreted medium. In the TLR4 experiment, MSCs were stimulated with an activated-MΦ medium. For this purpose, we activated spleen MΦs (from C57BL/6 male mice) with 100 ng/ml LPS (Sigma-Aldrich, St Louis, MO, USA) for 6 hours, then switched to a fresh medium and allowed them to secrete growth factors for 24 hours. Then, we treated the MSCs from both TLR4^-/- and WT mice with the activated-MΦ medium. Concentrations of cytokines were determined by Quasys Q-View™ imaging and a software system. For all multiplex assays, samples were run in triplicate wells.
Histopathological Evaluation

At the end of each study (7 or 28 days after cell transplantation), the hearts were perfused with 4% buffered formalin (Biolab, Jerusalem, Israel), harvested and sectioned into two transverse slices parallel to the atrioventricular ring. Each slice was embedded in paraffin, sectioned into 5-μm slices and stained with hematoxylin and eosin (H&E) or immunolabeled with specific antibodies.

Postmortem morphometric analysis was performed on recipient mice hearts 7 days after TLR4−/− deficient or WT MSC transplantation (8 days after MI). The hearts were perfused with 4% formaldehyde (15 mm Hg) for 20 minutes, and measurements were performed on slices obtained ~5 mm from the apex of the heart. The slides were stained with Masson's Trichrome staining and H&E, photographed, and analyzed with the use of planimetry software (Sigma Scan Pro version 5). We measured the average LV scar thickness (from 3 sites of scar), the relative LV scar area (scar area divided by muscle area) and the relative LV scar length (scar length divided by muscle length) in each heart.

To assess the effect of TLR4−/− cMSCs on macrophage accumulation in the infarct site, heart sections (one per mouse) were immunolabeled with rat anti-mouse F4/80 (AbD Serotec, Kidlington, Oxfordshire, UK).

Heart sections (from each mouse) were immunolabeled with rabbit anti-mouse CD47/MER6 (Bioss Antibodies, Woburn, MA, USA). To determine the survival of implanted male cMSCs from TLR4−/− or WT mouse, we stained the heart sections from female recipients with anti-mouse SRY (E-19) (sex-determining region Y protein) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and hematoxylin (for nuclear staining).
Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, USA). Variables are expressed as mean±SEM. Specific statistical tests are detailed in the Figure legends. In brief, differences between values were tested by unpaired t-test or one-way ANOVA test (> 2 groups). If values were not normally distributed (tested by the D’Agostino-Pearson omnibus normality test), we used the non-parametric Mann-Whitney test or Kruskal-Wallis test (> 2 groups), followed by the Dunn's multiple comparison post-test. Differences in levels of various cytokines were tested by a two-tailed unpaired multiple t-test followed by Holm-Sidak’s multiple comparison test. We used two-way repeated-measures ANOVA, and Holm Sidak’s multiple comparison test to assess the significance of predefined comparisons at specific time points. Differences in the CD47 expression were tested by 2-way ANOVA followed by Holm-Sidak’s test.

Results

Characterization of MSCs from the Failing Heart

Chronic heart failure and LVD are associated with low-grade inflammation.18, 19 To determine the effect of LVD on resident cMSCs and scMSCs, we isolated MSCs from female Balb/c mice with LVD, 28 days after extensive MI (n=9) or sham MI (sham) (n=7). LVD was determined by reduced LV ejection fraction, 28 days after MI, compared with sham operation (27.9%±3.8 vs. 54.9%±3.8, p<0.0001). We then isolated and cultured MSCs from both cardiac and fat tissue after LVD or sham operation, as previously described.3, 14

MSCs were defined according to the three criteria of the International Society for Cellular Therapy.16 Both cMSCs and scMSCs, isolated after either MI or sham operation, were
plastic-adherent and displayed a mesenchymal spindle shape (Figure 1A). We next evaluated cell proliferation rate for five days and found that cMSCs from the failing hearts had the highest growth rate compared with other groups (Figure 1B). Characterization of cardiac and scMSCs after MI or sham by flow cytometry showed that cultured cells from the different groups expressed different levels of mouse MSC markers, such as CD105, a part of the complex of transforming growth factor (TGF) -β receptor and a marker of MSC subset with reparative, myogenic properties. All were negative for the hematopoietic lineage markers: CD11b, CD45, CD31 and TER119 (Figure 1C). Finally, tri-lineage differentiation into osteoblasts, adipocytes and chondrocytes confirmed the identity of the MSCs (Figure 1D-F). Together, our findings indicate that LVD affects the phenotype of MSCs, particularly cMSCs.

LV Dysfunction modulates MSC secretome

MSCs modulate infarct repair through their paracrine, trophic, immune-modulating and anti-inflammatory properties. Therefore, we sought to determine the effect of LVD on the cytokine profile of MSCs. For that purpose, we measured the level of secreted cytokines in the conditioned medium of cultured MSCs. Significantly, cMSCs isolated from LVD secreted higher levels (~1.3 fold) of pro-inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor- α (TNF-α), compared with their sham counterparts (Figure 2A-D). The levels of the anti-inflammatory cytokines IL-10 and IL-4 remained unchanged (Figure 2E-F). Interestingly, the levels of the MΦ (macrophage)-recruitment factors: regulated on activation normal T-cell expressed and secreted (RANTES), MΦ inflammatory protein-1α (MIP1-α) and monocyte chemotactic protein-1 (MCP-1), were significantly increased in cMSCs from LVD (2.4-, 3.5- and 1.6-fold higher), compared with sham-isolated cMSCs (Figure 2G-I). Notably, LVD induced a different secretory profile in scMSCs (Figure 2). Most cytokines secreted from
scMSCs, such as TNF-α, IL-10, RANTES, MIP-1α and granulocyte macrophage colony-stimulating factor (GM-CSF), showed either a decrease (~0.6-fold lower) (Figure 2D-E, G-H and J) or no change in cytokine levels, compared with their sham counterparts (Figure 2A, B and F).

The secretion of IL-6, which acts as both a pro-inflammatory and reparative cytokine, was higher in both cMSCs (1.2-fold) and scMSCs (1.3-fold) from LVD mice, compared with sham mice (Figure 2C). Overall, while LVD switched cMSCs toward a pro-inflammatory phenotype, scMSCs were differently affected, by decreasing secretion of MΦ-recruitment and anti-inflammatory factors (Figure 2K).

**LVD reduced the favorable effects of cMSCs**

We next sought to determine whether LVD affects the reparative properties of MSCs in vivo. For that purpose, we isolated and expanded (3 passages) MSCs from cardiac or fat tissue of mice with either LVD or sham operation (Supplementary Figure 1). Then, we injected $10^5$ MSCs or saline (20μl) into the ischemic border zone of recipient mice immediately after MI, as previously described. Cardiac function was evaluated by echocardiography on day 1 and 28 days after MI and cell transplantation. Surprisingly, MSCs from all sources failed to improve cardiac function 28 days after MI, compared with saline (Supplementary Table 1 and Supplementary Figure 2). However, cMSCs from LVD worsened anterior wall thinning, compared with mice treated with scMSCs from LVD (Supplementary Table 1). This is significant because based on Laplace's Law, scar thinning increases wall stress, subsequent infarct expansion and LV remodeling.

Thus, our findings suggest that the inflammatory environment after MI restricts the therapeutic benefits of transplanted MSCs, and that pro-inflammatory, LVD-derived cMSCs might impair healing and worsen myocardial damage.
The role of TLR4 in cMSC polarization

To investigate the mechanism by which the inflammatory environment of LVD restricts the therapeutic benefit of cMSCs, we sought to test the hypothesis that TLR4 mediates cMSC pro-inflammatory polarization. The rationale for our hypothesis was that TLR4 is expressed by MSCs and could mediate the response to local inflammatory signals.9, 24 Thus, we isolated and characterized cells from the hearts of transgenic TLR4-/− mice (C57BL/10ScN) (n=5) and control WT (C57BL) mice (n=4) (Figure 3A). We then evaluated MSC proliferation rate for four consecutive days and found a lower proliferation rate in cMSCs from TLR4-/− mice, compared with WT MSCs (Figure 3B).

To characterize the phenotype of cMSCs, we used flow cytometry. There were no differences between TLR4+/− and WT cMSCs in the expression of mouse MSC markers (CD105 and CD90), and hematopoietic lineage markers (CD45, TER119 and CD31) (Figure 3C). Tri-lineage differentiation confirmed the identity of MSCs from both TLR-/− and WT MSCs (Figure 3D-F).

Notably, the secretome analysis of cMSCs after inflammatory stimulation revealed that TLR4 deficiency in cMSCs reduced their pro-inflammatory phenotype (Figure 4). To mimic the LVD or post-MI inflammatory environment, we challenged cultured cMSCs from TLR4+/− and WT mice with a medium of LPS-primed MΦs. We then analyzed the secreted cytokines in the conditioned media (Figure 4). Remarkably, TLR4 deficiency in cMSCs decreased the secretion of pro-inflammatory cytokines such as IL-1α, IL-12, IL-1β, and IL-6 (by 25%, 13%, 8% and 10%), compared with WT cMSCs (Figures 4A-D). On the other hand, the levels of IL-10, an anti-inflammatory cytokine, were increased by 23% in the TLR4+/− cMSCs, while IL-4 remained unchanged (Figure 4F-G). Furthermore, TLR4 deficiency in cMSCs increased MΦ-recruitment
chemokines such as GM-CSF and RANTES by 190% and 52%, compared with WT cMSCs (Figure 4H-I). Without MΦ stimulation, secretion levels were much lower without significant differences in the paracrine profile between the groups, except for an increase in IL-6, TNFα and MIP-1α levels, by 88%, 15%, and 93% in TLR4+/− cMSCs (Figure 4D, 4E and 4J). Together, our results indicate that TLR4 deficiency in cMSCs diminishes the inflammatory properties and increase some chemotactic properties in activated WT cMSCs. Our data suggest that TLR4 mediates, at least in part, cMSC polarization toward a pro-inflammatory phenotype in response to inflammatory signals, such as occur in LVD (Figure 4K).

**TLR4 deficiency in cMSCs improves their reparative properties in vivo**

To further assess the role of MSC -TLR4 in mediating the response of MSCs to local signals in the injured heart, we injected cMSCs from TLR4+/− or WT male mice into the hearts of recipient female mice subjected to acute MI. The efficacy of MSC transplantation from gender and strain mismatch was confirmed in a separate experiment (Supplementary Figure 3). We evaluated cardiac function and remodeling by echocardiography at 1 and 7 days after cell transplantation. The rationale for a 7-day follow-up was to determine the effect of local inflammation on the function of transplanted cells. Significantly, TLR4+/− cMSCs were more effective than WT MSCs in the prevention of adverse LV dilatation after MI (Figure 5 and Supplementary Table 2). Compared with WT cMSCs and saline, TLR4+/− cMSCs decreased the changes in LV diastolic diameter by 2.4- and 2.9-fold respectively (Figure 5A), and LV diastolic volume by 2.5- and 3-fold, respectively (Figure 5C). Thus, deficiency of TLR4 in cMSCs enhanced their ability to improve cardiac remodeling after MI.

Postmortem morphometry revealed that scar length and area were smaller, and scar thickness was greater in hearts treated with TLR4+/− cMSCs, compared with controls, 7 days after
MI (Figure 6A-F). By microscopic examination, infarcts treated with WT MSCs displayed extensive inflammation at the infarct site (Figure 6B), while the saline-treated group showed a thinner, fibrotic scar, 7 days after MI (Figure 6C). These findings probably reflect an immune response against the implanted cMSCs. Of interest, a microscopic evaluation revealed islands of karyolitic (loss of the nuclei) cardiomyocytes in the infarct zone of hearts treated with TLR4\(^{-/-}\) cMSCs, 7 days after MI. These islands were characterized by cardiomyocyte hypereosinophilia and absence of nuclei and striation (Figure 6A). The area of the karyolitic islands in the TLR4\(^{-/-}\) cMSC-treated mice was larger, compared with WT MSCs and saline-treated groups (Figure 6G).

In the experiments with TLR4\(^{-/-}\) and WT cMSCs, we transplanted cells from male donor to female recipient mice hearts. Thus, we were able to assess cell retention by staining for the sex-determining region Y (SRY) chromosome (Figure 7A-C). Indeed, we identified significant amounts of donor cells at the site of transplantation of TLR4\(^{-/-}\) cMSCs, around the karyolitic myocardial islands, 7 days after injection (Figure 7A1-3). In contrast, we identified only a few donor cells in the WT cMSC-treated hearts (Figure 7B1-3).

Interestingly, we identified numerous macrophages and cells expressing CD47 around the karyolitic islands (Figure 7D-E). CD47 is a marker of cell viability and communicates anti-phagocytic “don’t eat me” signals to M\(\Phi\)s.\(^ {25-27}\) To understand the interaction between TLR4 and CD47, we analyzed cMSCs from TLR4\(^{-/-}\) and WT by flow cytometry. CD47 expression was abundant on both WT and TLR4\(^{-/-}\) cMSCs in vitro (Figure 7F). However, exposure of cMSCs to 10 ng/ml of LPS (TLR4 agonist) for 20 hours, significantly down-regulated CD47 expression in WT, but not in TLR4\(^{-/-}\) cMSCs (Figure 7F). The minimal LPS dose used here mimicked the gradient of danger signals from the injury site,\(^ {9}\) and switched MSCs into a pro-inflammatory phenotype.\(^ {9}\) Interestingly, a high dose of the TLR4 agonist (100ng/ml) did not affect the
expression of CD47 on either WT or TLR4−/− cMSCs (Figure 7F), probably because of TLR4 down-regulation as a negative feedback mechanism.9 Together, our findings indicate that TLR4 deficiency in cMSCs preserves the expression of CD47 on implanted cMSCs, thus improving the survival of implanted MSCs and delaying phagocytosis of ischemic cardiomyocytes, which subsequently leads to increased scar thickness and attenuation of LV dilatation.

**Discussion**

Our study suggests that LVD modulates the phenotype and function of MSCs from cardiac tissue, and to a lesser extent from sc fat, and attenuates their reparative properties. This is important because pro-inflammatory MSCs can aggravate inflammation and myocardial damage, either directly or by influencing the immune response.4, 6 Transplantation of MSCs from LVD or sham into the inflammatory environment after MI did not improve cardiac remodeling and function. Moreover, pro-inflammatory cMSCs from LVD exacerbated scar thinning after MI. The pro-inflammatory polarization of cMSCs was mediated by TLR4 as TLR4 deficiency attenuated pro-inflammatory MSC polarization and improved their ability to reduce early cardiac remodeling after MI. Our findings could be relevant to the clinical applications of MSC-based therapy and help guide the selection of MSC source when deciding between autologous vs. donor MSCs for cell-based therapy. To the best of our knowledge, our study is the first to show the potential negative interaction between cMSCs and the failing or infarcted heart, and the key role of TLR4 in pro-inflammatory polarization of MSCs, in the setting of LVD.

The retention and survival of MSCs in the heart are limited,2, 28 and their favorable effects are attributed to “hit and run” paracrine effects.6 The favorable effect of implanted MSCs upon resident cMSCs, MΦs,14 endothelial,3 and cardiac stem cells,29 is largely paracrine. In our study,
the improved retention of TLR4<sup>−/−</sup> cMSCs could contribute to improved infarct repair. The poor survival of implanted WT MSCs could explain the rarity of reported adverse effects in clinical trials. Our findings could be significant for the new tissue engineering approaches to increase cell retention and survival in the injured heart, such as injectable biomaterials, scaffolds, and microspheres. With greater MSC retention and survival, however, we might see more adverse effects.

Our results are in line with the new paradigm of MSC polarization<sup>3,9</sup> Waterman et al.<sup>9</sup> showed that MSCs can polarize into two functional phenotypes following specific TLR stimulation, resulting in different immune modulatory effects and secretomes, <i>in vitro</i>. While the TLR4-primed MSCs develop pro-inflammatory characteristics (MSC1), the TLR3-primed MSCs develop an anti-inflammatory profile (MSC2).

The present work confirms and extends our previous work and that of others<sup>3, 7, 30, 31</sup> suggesting that MSCs from different tissues have a different phenotype, proliferation rate, and function. Significantly, the present study and our previous work with human MSCs<sup>3</sup> suggest that heart disease affects the phenotype and function of MSCs from different tissues.

**The role of TLR4 in MSC polarization**

TLR4 emerged as a potential regulator that could mediate MSC pro-inflammatory polarization. TLRs play a critical role in the activation of innate and adaptive immunity<sup>32</sup> and trigger inflammation leading to dysfunctional wound healing in several chronic diseases<sup>33</sup>. Loss of TLR4 provides protection against ischemia/reperfusion injury and post-MI remodeling<sup>34, 35</sup>. However, it is unclear which cardiac cell is responsible for such protection. Importantly, not only immune cells express TLR, but a variety of other cell types do, including MSCs<sup>3, 24, 36</sup>. The activation of the TLR4 pathway stimulates secretion of pro-inflammatory mediators by MSCs<sup>9</sup>.
In the setting of acute or chronic myocardial injury, TLR4 can be activated by endogenous ligands called damage-associated molecular patterns (DAMPs). These endogenous activators, such as heat shock proteins, are derived from dying cells, and can promote chronic heart failure.

The present study shows that MSCs from TLR4−/− mice, but not from WT mice, improve early LV remodeling after MI. Moreover, transplanted TLR4−/− cMSCs increased scar thickness. This is important because based on Laplace's law, increased scar thickening reduces wall stress, and subsequent infarct expansion and LV remodeling. Indeed, implanted TLR4−/− cMSCs attenuated LV dilatation after MI.

An unusual finding was the presence of islands of karyolitic cardiomyocytes surrounded by MΦs in the infarcts of mice treated with TLR4−/− cMSCs. MSCs regulate MΦ polarization, and inhibit the phagocytic capacity of MΦs. The inhibitory effect of TLR4−/− cMSCs upon MΦ phagocytic capacity could partially explain the unique finding of karyolitic cardiomyocyte islands. Interestingly, the cells that encircled the islands of karyolitic cardiomyocytes expressed CD47, a marker of “don’t eat me” signal, which prevents phagocytosis and is associated with improved myocardial protection and anti-fibrotic effects on the heart. We have shown that while a low dose of the TLR4 agonist LPS, which mimics chronic inflammation, down-regulates CD47 expression in MSCs, TLR4 deficiency preserves it. Maintenance of CD47 expression could be an advantage to avoid a “clean-up” response by cardiac MΦs. It seems that CD47 expression improves implanted MSC survival, delays cardiomyocyte phagocytosis, increases scar thickness, and enhances a short-term therapeutic effect. Nevertheless, the significance of MSC CD47 expression and delayed phagocytosis after TLR4−/− MSC therapy needs a longer...
follow-up period, since inefficient clearance of dying cardiomyocytes has been associated with suboptimal tissue remodeling after MI.45

Taken together, our study proposes that TLR4 deficiency in resident and transplanted MSCs could diminish the negative effects of cardiac inflammation on the MSC’s reparative properties, create an improved environment for MSC retention and improve the outcome of MSC therapy after MI (Figure 8).

Limitations

First, characteristics of MSCs were based on cultured cells. Cell culture duration and the degree of cell expansion have an impact on MSC phenotype and functional properties.6 Nevertheless, MSCs from different sources were cultured under identical conditions, and our findings clearly demonstrated the differences in characteristics and function between MSCs from LVD compared with controls. Second, cMSCs are a heterogeneous cell population, derived from various sources. Thus, the diverse origins for subpopulations of cMSCs and the influence on their ability for tissue repair warrant further research. Third, to verify similar myocardial injury among the experimental groups, we performed the first echocardiography study after MI. However, a pre-injury echocardiography is warranted to confirm similar baseline parameters among experimental groups. Fourth, although we used a low level of anesthesia, echocardiography in conscious mice could avoid the adverse effects of anesthesia and may produce more physiological results.46 Finally, seven days of follow-up in the experiment of TLR4−/− cMSC transplantation might be too short; the beneficial effects of TLR4−/− cMSCs could disappear during a longer follow-up period.

Summary, implications and future research

Our study shows that the environment of the failing and infarcted myocardium drives resident
and transplanted MSCs toward a pro-inflammatory phenotype, and restricts their reparative properties in a mechanism mediated by TLR4. Further understanding of the molecular mechanisms that govern the interactions between MSCs and inflammation is essential for developing new MSC-based therapies, and for improving our knowledge of myocardial inflammation and fibrosis. The ability to improve infarct healing and treat LVD by optimizing MSC therapy, could help us to advance the field of cardiovascular regenerative medicine, particularly in aged and chronically ill patients whose MSC regenerative capacity could be impaired.

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Disclosures

None
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Figure Legends

Figure 1. Characterization of MSCs from LVD mice.

A. Morphology of cells isolated from the heart and sc fat of mice after MI or sham operation.
Cells were plastic-adherent and displayed a mesenchymal spindle shape. Representative pictures from each group at passage 1.

B. Growth of cMSCs and scMSCs from mice with LVD, 28 days after MI, or mice after sham operation. Proliferation rate was evaluated after 3 passages based on an optical density measurement by XTT reaction for 5 consecutive days in each MSC group. cMSCs from LVD mice displayed the highest growth rate (at 96 hours, \( p < 0.001 \)). Results are expressed as the mean optical density for each day per MSC group ±SEM. Measures of cell proliferation over time and among groups were analyzed by a 2-way repeated-measures ANOVA and Holm-Sidak’s multiple comparison test.

C. Immune-phenotyping of cells isolated from the heart and sc fat of LVD or sham mice by flow cytometry analysis. Cultured cells from the different groups expressed diverse levels of mouse MSC markers (CD105 and CD90), but all were negative for the hematopoietic lineage markers (CD11b, CD45, CD31 and TER119). Results are expressed as percentage (mean± SEM) of positive cells of the total number of cells analyzed. Measures were analyzed for each marker between the groups by a 1-way ANOVA and Holm-Sidak’s multiple comparison test.

D-F. Multi-lineage differentiation capacity of MSCs from the heart and SC fat of LVD or sham mice by in vitro differentiation assays. Representative MSCs from each group induced to differentiate into osteogenic, adipogenic and chondrogenic lineages. Overall, the different MSCs exhibit similar differentiation capacity. Positive staining for Alizarin Red S (D) indicates osteogenic differentiation, compared with untreated MSCs. Positive staining for Oil Red O (E)
indicates adipogenic differentiation, compared with untreated MSCs. Positive staining for Alcian Blue (F) indicates condrogenic differentiation.

Abbreviations: cMSCs = cardiac mesenchymal stromal cells; LVD= left ventricular dysfunction; MI = myocardial infarction; MSCs = mesenchymal stromal cells; sc = subcutaneous.

Figure 2. The effect of LVD on the paracrine profile of MSCs.

A-J. To evaluate the effect of LVD on MSC cytokine profiles, we measured the levels of secreted cytokines in the conditioned medium collected from the cells following LVD, and compared them with their sham counterparts by Multiplex ELISA. cMSCs and scMSCs from LVD mice significantly changed their paracrine profile, compared with their sham counterparts. While LVD cMSCs became more inflammatory (A-D), scMSCs were differently affected, and mainly decreased their anti-inflammatory (E) and macrophage-recruitment factors (G, H and J). Results are presented as the mean of cytokine secretion levels (pg/ml) ±SEM. Dotted line indicates the basal level of each cytokine in the growth medium. P values addressing differences in cytokine secretion were calculated by a multiple two-tailed unpaired t-test followed by Holm-Sidak’s multiple comparison test. The * indicates a statistically significant p value by Holm-Sidak’s test.

K. Fold change of cytokine secretion from MSCs derived from LVD, compared with sham-operated mice. While LVD drives cMSCs toward a pro-inflammatory phenotype, scMSCs were affected differently, and mainly decreased their MΦ-recruitment and anti-inflammatory factors. Fold change was calculated as the ratio between cytokine levels in LVD and sham MSCs. The * indicates a statistically significant p value by Holm-Sidak’s test.
Abbreviations: cMSCs = cardiac mesenchymal stromal cells; GM-CSF = granulocyte macrophage colony-stimulating factor; IL = interleukin; LVD= left ventricular dysfunction; МΦ = macrophage; MCP-1 = monocyte chemotactic protein-1; MI = myocardial infarction; MIP-1α = macrophage inflammatory protein-1α; MSCs = mesenchymal stromal cells; RANTES = regulated on activation, normal T cell expressed and secreted; sc = subcutaneous; TNF-α = tumor necrosis factor-α.

Figure 3. Characterization of cMSCs from TLR4−/− and WT mice.

A. Plastic-adherent cMSCs isolated from the hearts of TLR4−/− and WT mice. Representative images of cells after one passage.

B. Growth curves of cMSCs isolated from the hearts of TLR4−/− (n=4) and WT (n=4) mice. At second passage, proliferation rate was evaluated based on optical density measurement by XTT reaction for four consecutive days in both MSC groups. TLR4−/− cMSCs displayed a slower growth rate (p<0.01 after 48 hours and p<0.05 after 72 hours). Results are expressed as the mean optical density for each day per MSC group ±SEM. Measures of cell proliferation over time and among groups were analyzed by a 2-way repeated-measures ANOVA and Holm-Sidak’s multiple comparison test.

C. Phenotype of cells isolated from the heart of TLR4−/− (n=3) and WT (n=3) mice by flow cytometry analysis. No differences were found between the groups in expression levels of mouse MSC markers (CD105 and CD90). Results are expressed as percentage (mean± SEM) of positive cells of the total number of cells analyzed. Since data are not normally distributed (tested with D’Agostino & Pearson omnibus normality test), p values were calculated for each marker between TLR4−/− and WT by non-parametric Mann-Whitney test.
D-F. Multi-lineage differentiation capacity of cMSCs from of TLR4<sup>−/−</sup> and WT mice by in vitro differentiation assays. Representative MSCs from each group induced to differentiate into osteogenic, adipogenic and chondrogenic lineages. Positive staining for Alizarin Red S (D) indicates osteogenic differentiation. Positive staining for Oil Red O (E) indicates adipogenic differentiation. Positive staining for Alcian Blue (F) indicates condrogenic differentiation. cMSCs from TLR4<sup>−/−</sup> exhibited greater differentiation capacity toward bone but less toward cartilage, compared with WT cMSCs. Adipogenic capacity was similar between groups.

**Abbreviations:** cMSCs = cardiac mesenchymal stromal cells; MSC = mesenchymal stromal cell; ns = not significant; TLR4 = toll-like receptor 4; WT = wild type.

Figure 4. TLR4 deficiency in cMSCs decreases the secretion of pro-inflammatory cytokines.

A-I. To determine the effect of TLR4 deficiency on the cytokine secretion profile of cMSCs, we measured the levels of secreted cytokines in the conditioned medium collected from cMSCs from TLR4<sup>−/−</sup> and WT mice by Multiplex ELISA, after induction with activated-MΦ medium. Overall, TLR4 deficiency in cMSCs decreased the secretion of inflammatory cytokines (A-D) after stimulation with activated-MΦ medium, whereas the levels of the anti-inflammatory cytokines (F-G) were increased in the TLR4<sup>−/−</sup> cMSCs. The lack of TLR4 in cMSCs also increased MΦ-recruitment factors (H-J) after stimulation with activated-MΦ medium. Results are presented as the mean of cytokine secretion levels (pg/ml) ±SEM. Solid line indicates the level of cytokine secreted by the LPS activated-MΦ and dashed line indicates the level of cytokine in the growth medium. *P* values addressing differences in cytokine secretion were calculated by a multiple two-tailed unpaired t-test followed by Holm-Sidak’s multiple comparison test. The * indicates a statistically significant *p* value by Holm-Sidak’s test.
K. Fold change of cytokine secretion from cMSCs with and without \textit{TLR4} deficiency, after stimulation with activated MΦ medium. Loss of \textit{TLR4} in cMSCs resulted in decreased secretion of pro-inflammatory cytokines and increased secretion of some MΦ-recruitment and anti-inflammatory cytokines. Change in cytokine levels was calculated as the ratio of cytokine levels between \textit{TLR4}^{-/-} and WT cMSCs. The * indicates a statistically significant \textit{p} value by Holm-Sidak’s test.

\textit{Abbreviations:} cMSCs = cardiac mesenchymal stromal cells; GM-CSF = granulocyte macrophage colony-stimulating factor; IL = interleukin; MΦ = macrophage; LPS = lipopolysaccharide; MCP-1 = monocyte chemotactic protein-1; MIP-1\textalpha = macrophage inflammatory protein-1\textalpha; RANTES = regulated on activation, normal T cell expressed and secreted; \textit{TLR4} = toll-like receptor 4; TNF-\textalpha = tumor necrosis factor-\textalpha; WT = wild type.

\textbf{Figure 5. Deficiency of \textit{TLR4} in implanted cMSCs improves their reparative properties and attenuates LV dilatation in recipient mice, 7 days after MI.}

To evaluate the impact of \textit{TLR4} deficiency on the reparative properties of cMSCs, we injected cMSCs isolated from \textit{TLR4}^{-/-} or WT mice into the hearts of recipient mice subjected to MI and measured their cardiac function. cMSCs (\textit{TLR4}^{-/-} and WT) or saline were injected into the ischemic zone immediately after MI. Echocardiography studies were done on days 1 and 7 after MI and cell or saline injection.

\textbf{A-D.} The graphs display the mean percentage of change \pm SEM in echocardiography measurements from day 1 to day 7. cMSCs from \textit{TLR4}^{-/-} mice improved LV diastolic and systolic diameter (\textbf{A-B}) and volume (\textbf{C-D}) compared to the saline-injected group (\textit{p}<0.05 in diastole). Change (\%) from day 1 value was calculated for each animal as follows: [(follow-up
value –day 1 value)/day 1 value] ×100. The difference between groups was tested by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test.

**Abbreviations:** cMSCs = cardiac mesenchymal stromal cells; LV = left ventricle; MI = myocardial infarction; MSCs = mesenchymal stromal cells; TLR4 = toll-like receptor 4; WT = wild type.

**Figure 6. Islands of karyolitic cardiomyocytes in the infarcted hearts of mice treated with TLR4−/− cMSCs, 7 days after MI.**

A-C. To determine the effect of TLR4−/− cMSCs on LV structure 7 days after MI, hearts were stained with Masson's Trichrome (A-C, left and middle columns) or H&E (A-C, right column). The infarct zone of the TLR4−/− cMSC-treated group contained islands of karyolitic cardiomyocytes (cardiomyocytes with loss of their nuclei), which were characterized by hypereosinophilia of cardiomyocytes (A, black arrows). On the other hand, hearts of mice treated with WT cMSCs displayed extensive inflammation in the infarct zone, demonstrated by immune cell infiltration (B, purple). The saline-treated group showed mostly scar formation (C).

D-G. Morphometric measurements of scar thickness (D), relative scar area (E) and relative scar length (F) revealed a smaller and thicker scar in the TLR4−/− cMSC- treated group, compared with WT MSCs and saline-treated groups. Morphometric measurements of the cardiomyocyte karyolitic islands (G) in mice hearts from the different groups showed significantly larger areas of karyolitic islands in hearts treated with TLR4−/− cMSC, compared with WT cMSC- and saline-treated groups. P value for the difference between groups was calculated by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test.
Abbreviations: cMSCs = cardiac mesenchymal stromal cells; H&E = hematoxylin and eosin; LV = left ventricle; MI = myocardial infarction; TLR4 = toll-like receptor 4; WT = wild type.

Figure 7. Outcome of the implanted cMSCs.

A-C1-3. Donor MSCs in the infarcted myocardium, 7 days after transplantation in mice. To determine the survival of implanted cMSCs (male) in infarcted myocardium (female), we stained the hearts against SRY, 7 days after transplantation. Representative microscopic images of the infarct zone: brown nuclear staining indicates transplanted, SRY positive cMSCs and purple nuclear staining indicates host cells. cMSCs were found at the border of the karyolytic sites and were abundant in the heart sections treated with TLR4−/− cMSCs (A-C3, red arrows).

D-E. Immunostaining of the karyolytic sites revealed large numbers of infiltrating cells at the border zone, some of them stained by the MΦ marker F4/80 (D) and some expressed the "don’t eat me" marker CD47 (E).

F. Evaluation of CD47 expression on cultured MSCs. Both WT and TLR4−/− cMSCs expressed CD47. Exposure of cMSCs to a low dose of LPS (10 ng/ml) down-regulated CD47 expression in WT, but not in TLR4−/− cMSCs. P value for the difference among the groups was calculated by 2-way ANOVA test followed by Holm-Sidak's multiple comparison test.

Abbreviations: cMSCs = cardiac mesenchymal stromal cells; LPS = lipopolysaccharide; SRY = sex-determining region Y; MI = myocardial infarction; TLR4 = toll-like receptor 4; WT = wild type.
Figure 8. A proposed model for TLR4-based “re-education” of resident and transplanted MSCs by the failing heart environment.

Resident and transplanted MSCs from cardiac patients are affected by local factors released by damaged cells during MI. These factors, also called DAMPs, are known to bind to TLR4, which together polarize the MSCs toward a pro-inflammatory phenotype and reduce their CD47 expression ("don’t eat me" signal), thereby impairing their reparative potential and survival. * indicates findings from previous study.$^3$

**Abbreviations:** DAMPs = danger-associated molecular patterns; LV = left ventricle; MΦ = macrophage; MSCs = cardiac mesenchymal stromal cells; sc = subcutaneous; TLR4 = toll-like receptor 4; WT = wild type.
A

**TLR4**

Masson's Trichrome x40

Masson's Trichrome x100

H&E x400

**Outcome:**

Karyolitic islands

B

**WT**

Masson's Trichrome x40

Masson's Trichrome x100

H&E x400

Inflammation

C

**Saline**

Masson's Trichrome x40

Masson's Trichrome x100

H&E x400

Scar formation

D

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G

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Left Ventricular Dysfunction Switches Mesenchymal Stromal Cells Toward an Inflammatory Phenotype and Impairs Their Reparative Properties via Toll-like Receptor-4
Nili Naftali-Shani, La-Paz Levin-Kotler, Dahlia Palevski, Uri Amit, David Kain, Natalie Landa, Edith Hochhauser and Jonathan Leor

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SUPPLEMENTAL MATERIAL

Left Ventricular Dysfunction Switches Mesenchymal Stromal Cells toward an Inflammatory Phenotype and Impairs their Reparative Properties via Toll-like Receptor-4

Running Title: Inflammatory Mesenchymal Stromal Cells

Nili Naftali-Shani, PhD\textsuperscript{1,2,3}; La-Paz Levin-Kotler, MSc\textsuperscript{1,2,3}; Dahlia Palevski, MSc\textsuperscript{1,2,3}; Uri Amit, MD, MPH, PhD\textsuperscript{1,2,3}; David Kain, PhD\textsuperscript{1,2,3}; Natalie Landa, PhD\textsuperscript{1,2,3}; Edith Hochhauser, PhD\textsuperscript{4} and Jonathan Leor, MD\textsuperscript{1,2,3}
## SUPPLEMENTAL TABLES

Supplementary Table 1: Effect of MSC therapy on LV remodeling and function by 2D-echocardiography, 1 and 28 days after MI.

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<thead>
<tr>
<th>Echo parameter</th>
<th>Days after MI and treatment</th>
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| Parameter                              | Value                  | Value                  | Value                  | Value                  | Value                  | Value                  | p value  \\
|----------------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|----------------------
| Ejection fraction (%)                  | 28                     | 27.1±8.1               | 24.6±6.2               | 31±6                   | 34.3±5.9               | 36.4±5                 |Interaction = 0.79 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.805                  | 0.805                  | 0.805                  | 0.805                  | 0.805                  | 0.805                  |                      \\
| LV diastolic area (mm²)                | 1                      | 9±1                    | 9±0.6                  | 9.9±0.5                | 9.9±0.4                | 10.3±0.5               |Treatment effect = 0.34 \p \\
| 28                                     | 14.4±2.2               | 11.8±1                 | 13.3±1.1               | 17.1±2.4               | 13.4±1.2               |                       | time <0.0001 \p interaction = 0.24 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.019                  | 0.066                  | 0.062                  | 0.0005                 | 0.022                  |                       |                      \\
| LV systolic area (mm²)                 | 1                      | 8±0.9                  | 7±0.6                  | 8±0.4                  | 7.9±0.3                | 7.5±0.6                |Treatment effect = 0.6 \p \\
| 28                                     | 11.3±2.5               | 9.3±1.1                | 9.8±1                  | 12.9±2.5               | 9.7±1.3                |                       | time =0.0002 \p interaction = 0.61 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.254                  | 0.276                  | 0.276                  | 0.025                  | 0.254                  |                       |                      \\
| Fractional area change (%)             | 1                      | 10.9±2.4               | 21.3±4.3               | 18.1±4.1               | 20.4±1                 | 27.7±3.1               |Treatment effect = 0.15 \p \\
| 28                                     | 24.4±5.8               | 21.3±4.2               | 26±4                   | 27.2±4.4               | 30.1±4                 |                       | time =0.01 \p interaction = 0.44 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.155                  | 0.997                  | 0.440                  | 0.547                  | 0.786                  |                       |                      \\
| Anterior wall diastolic thickness (mm) | 1                      | 0.73±0.03              | 0.96±0.06              | 0.89±0.07              | 0.8±0.05               | 0.87±0.05              |Treatment effect = 0.006 \p \\
| 28*                                    | *0.57±0.05             | *0.86±0.07             | 0.71±0.05              | 0.69±0.02              | 0.78±0.03              |                       | time =0.0007 \p interaction = 0.88 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.297                  | 0.317                  | 0.115                  | 0.317                  | 0.298                  |                       |                      \\
| Anterior wall systolic thickness (mm)  | 1                      | 0.82±0.03              | 1±0.06                 | 0.98±0.06              | 0.91±0.04              | 0.99±0.04              |Treatment effect = 0.01 \p \\
| 28                                     | 0.69±0.09              | 0.98±0.08              | 0.87±0.07              | 0.87±0.08              | 0.93±0.04              |                       | time =0.03 \p interaction = 0.95 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.694                  | 0.694                  | 0.694                  | 0.694                  | 0.694                  |                       |                      \\
| Posterior wall diastolic thickness (mm)| 1                      | 0.64±0.03              | 0.69±0.02              | 0.65±0.04              | 0.73±0.03              | 0.73±0.02              |Treatment effect = 0.09 \p \\
| 28                                     | 0.64±0.04              | 0.72±0.03              | 0.76±0.02              | 0.76±0.09              | 0.77±0.02              |                       | time =0.09 \p interaction = 0.68 \p \\
| p day 1 vs. 28 (Holm-Sidak)            | 0.955                  | 0.948                  | 0.191                  | 0.948                  | 0.796                  |                       |                      \\
| Posterior wall                         | 1                      | 0.7±0.04               | 0.86±0.04              | 0.81±0.07              | 0.88±0.03              | 0.91±0.03              |Treatment effect = 0.03 \p \\
|                                        |                        |                        |                        |                        |                        |                        | time =0.01 \p          |
Measurements of LV parameters, 1 and 28 days after MI and transplantation of cMSCs or scMSCs derived from LVD or sham mice. Values are mean±SEM. Probability values in the right column reflect comparison of the differences between the different treatment groups and their interactions over time by 2-way repeated-measures ANOVA followed by Holm-Sidak’s multiple comparison test. * $p = 0.036$; cMSCs vs. scMSCs from LVD mice. Probability values within the table represent comparison of the differences between measurements at day 1 and 28 in each treatment group by Holm-Sidak’s multiple comparison test.

**Abbreviations**: 2D = 2-dimensional; AW d = anterior wall diastolic thickness; AW s = anterior wall systolic thickness; cMSCs = cardiac mesenchymal stromal cells; LV = left ventricle; LVD = left ventricular dysfunction; MI = myocardial infarction; RM = repeated measure; scMSCs = subcutaneous MSCs.
Supplementary Table 2: Effect of TLR4<sup>−/−</sup> cMSC therapy on LV remodeling and function by 2D-echocardiography, 1 and 7 days after MI.

<table>
<thead>
<tr>
<th>Echo parameter</th>
<th>Time</th>
<th>TLR4&lt;sup&gt;−/−&lt;/sup&gt; cMSCs&lt;sup&gt;n=8&lt;/sup&gt;</th>
<th>WT cMSCs&lt;sup&gt;n=7&lt;/sup&gt;</th>
<th>Saline&lt;sup&gt;n=6&lt;/sup&gt;</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LV diastolic dimension (mm)</strong></td>
<td>Day 1 after MI</td>
<td>3.39±0.1</td>
<td>3.21±0.1</td>
<td>3.17±0.1</td>
<td>( p ) interaction = 0.05 ( p ) time &lt;0.0001 ( p ) treatment effect = 0.98</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>3.64±0.1</td>
<td>3.78±0.1</td>
<td>3.88±0.1</td>
<td></td>
</tr>
<tr>
<td>( p ) day 1 vs. 7 (Holm-Sidak)</td>
<td></td>
<td>0.048</td>
<td>0.0006</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td><strong>LV systolic dimension (mm)</strong></td>
<td>Day 1 after MI</td>
<td>2.67±0.1</td>
<td>2.67±0.2</td>
<td>2.6±0.1</td>
<td>( p ) interaction = 0.17 ( p ) time = 0.0004 ( p ) treatment effect = 0.72</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>2.87±0.1</td>
<td>3.12±0.2</td>
<td>3.31±0.1</td>
<td></td>
</tr>
<tr>
<td>( p ) day 1 vs. 7 (Holm-Sidak)</td>
<td></td>
<td>0.235</td>
<td>0.045</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td><strong>Fractional shortening (%)</strong></td>
<td>Day 1 after MI</td>
<td>21.36±4.1</td>
<td>17.55±3.3</td>
<td>18.25±3.1</td>
<td>( p ) interaction = 0.66 ( p ) time = 0.54 ( p ) treatment effect = 0.47</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>21.2±2.9</td>
<td>17.88±2.7</td>
<td>14.79±1.6</td>
<td></td>
</tr>
<tr>
<td>( p ) day 1 vs. 7 (Holm-Sidak)</td>
<td></td>
<td>0.995</td>
<td>0.995</td>
<td>0.631</td>
<td></td>
</tr>
<tr>
<td><strong>LV diastolic volume (mm&lt;sup&gt;3&lt;/sup&gt;)</strong></td>
<td>Day 1 after MI</td>
<td>47.93±4.5</td>
<td>42.85±6.8</td>
<td>40.7±4.4</td>
<td>( p ) interaction = 0.06 ( p ) time &lt;0.0001 ( p ) treatment effect = 0.99</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>56.86±4.5</td>
<td>62.20±4.9</td>
<td>65.89±5.7</td>
<td></td>
</tr>
<tr>
<td>( p ) day 1 vs. 7 (Holm-Sidak)</td>
<td></td>
<td>0.202</td>
<td>0.024</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td><strong>LV systolic volume (mm&lt;sup&gt;3&lt;/sup&gt;)</strong></td>
<td>Day 1 after MI</td>
<td>27.77±4.5</td>
<td>28.95±6.7</td>
<td>25.47±3.7</td>
<td>( p ) interaction = 0.15 ( p ) time = 0.0009 ( p ) treatment effect = 0.64</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>32.6±3.9</td>
<td>39.98±5</td>
<td>45.42±5.2</td>
<td></td>
</tr>
<tr>
<td>( p ) day 1 vs. 7 (Holm-Sidak)</td>
<td></td>
<td>0.334</td>
<td>0.093</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td><strong>Ejection fraction (%)</strong></td>
<td>Day 1 after MI</td>
<td>42.61±6.8</td>
<td>36.75±6.3</td>
<td>38.27±5.6</td>
<td>( p ) interaction = 0.61 ( p ) time = 0.55 ( p ) treatment effect = 0.51</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>42.98±5</td>
<td>37.2±4.9</td>
<td>31.75±3.2</td>
<td></td>
</tr>
<tr>
<td>( p ) day 1 vs. 7 (Holm-Sidak)</td>
<td></td>
<td>0.995</td>
<td>0.995</td>
<td>0.631</td>
<td></td>
</tr>
<tr>
<td><strong>LV diastolic area (mm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>Day 1 after MI</td>
<td>9.34±0.5</td>
<td>8.66±0.8</td>
<td>8.41±0.5</td>
<td>( p ) interaction = 0.58 ( p ) time &lt; 0.0001 ( p ) treatment effect = 0.83</td>
</tr>
<tr>
<td></td>
<td>Day 7 after MI</td>
<td>11.66±0.7</td>
<td>11.63±0.5</td>
<td>11.68±0.5</td>
<td></td>
</tr>
</tbody>
</table>
Measurements of LV parameters, 1 and 7 days after MI and transplantation of cMSCs from *TLR4<sup>−/−</sup>* or WT mice. Values are mean±SEM. Probability values in the right column reflect comparison of the differences between the different treatment groups and their interactions.
over time by 2-way repeated-measures ANOVA followed by Holm-Sidak’s multiple comparison test. Probability values within the table represent comparison of the differences between measurements at day 1 and 7 in each treatment group by Holm-Sidak’s multiple comparison test.

**Abbreviations:** 2D = 2-dimensional; cMSCs = cardiac mesenchymal stromal cells; LV = left ventricle; MI = myocardial infarction; RM= repeated measure; *TLR4* = toll-like receptor 4; WT = wild type.
Supplementary Figure 1: Study design. To evaluate the effect of MI and LVD on mouse resident MSCs, we isolated cells from the heart and subcutaneous fat of mice with LVD, 28 days after extensive MI, or sham operation. We characterized the different MSCs in vitro (passage 3), by flow cytometry, cytokine array, and tri-lineage differentiation potential. We next assessed their therapeutic potential in vivo by injecting them into the hearts of recipient mice subjected to acute MI.

Abbreviations: cMSCs = cardiac mesenchymal stromal cells; LVD = left ventricular dysfunction; MI = myocardial infarction; sc = subcutaneous.
Supplementary Figure 2: Percentages of change in LV parameters, 28 days after transplantation of MSCs from LVD or sham mice. Percent of change in LV parameters (A-J) was calculated for each animal as follows: [(day 28 parameter−day 1 parameter)/day 1 parameter] ×100. Values are mean±SEM. P values reflect the differences in change between the groups in each cardiac parameter. Since data are not normally distributed (tested with D'Agostino & Pearson omnibus normality test), p values were calculated by Kruskal Wallis with Dunn's multiple comparison test.
**Abbreviations:** 2D = 2-dimensional; cMSCs = cardiac MSCs; LV = left ventricle; LVD = left ventricular dysfunction; MI = myocardial infarction; MSCs = mesenchymal stromal cells; scMSCs = subcutaneous fat MSCs.
Supplementary Figure 3: Survival of mCherry-labeled MSCs from male C57BL/6 mice in infarcted myocardium of female Balb/C mice, 7 days after MI and MSC-therapy.

To confirm the survival of MSCs from a different gender and strain, we injected mCherry-labeled MSCs from male C57BL/6 mice into the infarcted heart of female Balb/C mice (n=3), immediately after MI. A. The injected MSCs were permanently labeled by infection with a pQC-mCherry retroviral vector. B. Representative microscopic images of heart sections, 7 days after MI and injection of mCherry MSCs. Red staining indicates transplanted mCherry-labeled MSCs (arrows) and blue indicates nuclear staining. Seven days after MI, we identified implanted mCherry-labeled MSCs at the infarct zone but not in the saline-treated hearts (C).

Abbreviations: LV = left ventricle; MI = myocardial infarction.
SUPPLEMENTAL REFERENCES