Ly-6C<sup>high</sup> Monocytes Depend on Nr4a1 to Balance Both Inflammatory and Reparative Phases in the Infarcted Myocardium

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**Rationale:** Healing after myocardial infarction involves the biphasic accumulation of inflammatory lymphocyte antigen 6C (Ly-6C)<sup>high</sup> and reparative Ly-6C<sup>low</sup> monocytes/macrophages (Mo/MΦ). According to 1 model, Mo/MΦ heterogeneity in the heart originates in the blood and involves the sequential recruitment of distinct monocyte subsets that differentiate to distinct macrophages. Alternatively, heterogeneity may arise in tissue from 1 circulating subset via local macrophage differentiation and polarization. The orphan nuclear hormone receptor, nuclear receptor subfamily 4, group a, member 1 (Nr4a1), is essential to Ly-6C<sup>low</sup> monocyte production but dispensable to Ly-6C<sup>high</sup> macrophage differentiation; dependence on Nr4a1 can thus discriminate between systemic and local origins of macrophage heterogeneity.

**Objective:** This study tested the role of Nr4a1 in myocardial infarction in the context of the 2 Mo/MΦ accumulation scenarios.

**Methods and Results:** We show that Ly-6C<sup>high</sup> monocytes infiltrate the infarcted myocardium and, unlike Ly-6C<sup>low</sup> monocytes, differentiate to cardiac macrophages. In the early, inflammatory phase of acute myocardial ischemic injury, Ly-6C<sup>high</sup> monocytes accrue in response to a brief C–C chemokine ligand 2 burst. In the second, reparative phase, accumulated Ly-6C<sup>high</sup> monocytes give rise to reparative Ly-6C<sup>low</sup> F4/80<sup>high</sup> macrophages that proliferate locally. In the absence of Nr4a1, Ly-6C<sup>high</sup> monocytes express heightened levels of C–C chemokine receptor 2 on their surface, avidly infiltrate the myocardium, and differentiate to abnormally inflammatory macrophages, which results in defective healing and compromised heart function.

**Conclusions:** Ly-6C<sup>high</sup> monocytes orchestrate both inflammatory and reparative phases during myocardial infarction and depend on Nr4a1 to limit their influx and inflammatory cytokine expression. (Circ Res. 2014;114:1611-1622.)

**Key Words:** hormone receptors, nuclear ▲ macrophages ▲ monocytes ▲ myocardial infarction

Myocardial infarction (MI) is a leading cause of death worldwide. Although the mortality rate from MI has declined steadily for the past 50 years because of the application of medical innovations, scientific discoveries, and improvements in public health, the total number of deaths is on the rise. The healing of acute ischemic myocardial injury influences the outcomes postinfarction decisively. Adverse remodeling of the infarcted left ventricle associates with the development of heart failure because of systolic dysfunction and ischemic mitral regurgitation, whereas absence of excessive expansion of the infarcted left ventricle associates with better long-term prognosis. Recent experimental work suggests that this critical healing process requires a precise balance between removal of debris and regulation of scar formation. Macrophages may contribute essentially to the healing and regenerative process, but their polyfunctionality requires caution. On the one hand, macrophage depletion in infarcted hearts impairs collagen deposition, necrotic cell clearance, and angiogenesis, predisposing to cardiac rupture and death. On the other hand, macrophage-induced inflammation can...
be harmful and cause post-MI heart failure. The molecular pathways that balance inflammatory and reparative macrophage functions, therefore, comprise novel potential targets of therapeutic intervention after MI.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Ccr</td>
<td>C-C chemokine receptor</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>ED</td>
<td>end-diastolic</td>
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<td>ES</td>
<td>end-systolic</td>
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<td>LV</td>
<td>left ventricular</td>
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<td>Ly-6C</td>
<td>lymphocyte antigen 6C</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>Mo/MΦ</td>
<td>monocyte/macrophage</td>
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<td>Nr4a1</td>
<td>nuclear receptor subfamily 4, group a, member 1</td>
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<td>WT</td>
<td>wild-type</td>
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Shortly after onset of ischemia, endothelial cells augment adhesion molecule expression that, along with released chemokines, trigger leukocyte mobilization and extravasation. Within days, numerous leukocytes of medullary and extramedullary origins accumulate in the infarcting myocardium in massive numbers. Among them, phagocytic monocyte-derived macrophages digest dead tissue, effector cytokine, influence collagen deposition, and promote angiogenesis. Optimal healing of the myocardium involves the biphasic accumulation of myeloid cells, expressing the surface glycoprotein lymphocyte antigen 6C (Ly-6C): inflammatory Ly-6Chigh monocytes/macrophages (Mo/MΦ) accumulate early, whereas reparative Ly-6C low Mo/MΦ accumulate later. It is unclear, however, whether the biphasic response occurs via the sequential recruitment of different monocyte subsets, which then differentiate to various macrophage subsets, as has been proposed in the MI setting, or whether the response evolves via local differentiation and polarization of macrophages, as has been shown in other situations.

Distinguishing between these scenarios has been difficult to address given the obstacles in tracking monocytes influx and macrophage differentiation in vivo.

The orphan nuclear hormone receptor nuclear receptor subfamily 4, group a, member 1 (Nr4a1) has gained attention as a molecular switch that controls many cellular functions. The orphan nuclear hormone receptor nuclear receptor subfamily 4, group a, member 1 (Nr4a1) has gained attention as a molecular switch that controls many cellular functions.16–19 Recent studies have shown that Nr4a1 is essential to the differentiation of monocyte subsets or via the local differentiation of macrophages. We reasoned that if the sequential recruitment hypothesis adequately explains the biphasic response, then the infarcted myocardium should lack Ly-6C low macrophages in Nr4a1−/− mice. If, however, the biphasic response occurs via local macrophage differentiation and polarization, then Ly-6C low macrophages should accumulate regardless of Nr4a1 expression. Therefore, studying the inflammatory and healing response in the context of Nr4a1 provided the double advantage of determining the role of Nr4a1 in MI, which is unknown, while also addressing whether the biphasic response after MI occurs via the sequential differentiation of monocyte subsets or via the local differentiation of macrophages.

**Methods**

For further details, see Material in Online Data Supplement.

**Animals and Animal Experiments**

Female C57BL/6J (wild-type [WT]), B6.SJL-Ptprc Peprb/B0Y (cluster of differentiation [CD] 45.1+), and C57BL/6-Tg(UBC-green fluorescence protein [GFP])30Scha/J mice (GFP+ were purchased from the Jackson Laboratory (Bar Harbor, ME). Nr4a1-deficient mice (Nr4a1−/−) were kindly provided by Cathrine C. Hedrick, La Jolla Institute for Allergy and Immunology, La Jolla, CA. All protocols were approved by the Animal Review Committee at Massachusetts General Hospital. C57BL/6J were lethally irradiated and reconstituted with WT and Nr4a1−/− bone marrow to generate respective chimeric mice. MI was induced by permanent ligation of the left anterior descending artery. For adoptive transfer studies, monocyte subsets were sorted from blood and spleens of CD45.2+ GFP+ mice and injected into CD45.1+ mice on day 3 after MI.

**Histology**

Murine hearts were embedded in Tissue-Tek O.C.T compound (Sakura Finetek) and paraffin for sectioning and staining.

**Flow Cytometry and Flow-Assisted Cell Sorting**

Antibodies used for flow cytometry are listed in the Material in Online Data Supplement. Data were acquired on a BD LSRII and analyzed with FlowJo. Cells were sorted with BD FACS AriaII.

**Reverse Transcription Polymerase Chain Reaction**

RNA was isolated from sorted cells with the RNeasy Micro Kit (Qiagen). Quantitative real-time TaqMan polymerase chain reaction was run on a 7500 Polymerase Chain Reaction thermal cycler (Applied Biosystems).

**Echocardiography**

Images were acquired with a 13-MHz linear-array transducer (Vivid 7; GE Medical Systems) and analyzed with EchoPacs (GE Medical System). Fractional shortening was calculated using end-diastolic (ED) and end-systolic left ventricular (LV) inner diameters as fractional shortening=(LV inner diameter−LV inner diameter)/LV inner diameter×100%. LV ejection fraction was calculated using ED and end-systolic volumes as LV ejection fraction=(LVED volume−LV end-systolic volume)/LVED volume×100%.

**Statistics**

Results are shown as mean±SEM. The unpaired Student t test was applied to evaluate differences between 2 study groups. One-way ANOVA with post hoc Tukey multiple comparisons test was performed when comparing ≥2 groups. P values of ≤0.05 denote significant changes.

**Results**

**Nr4a1-Expressing Myeloid Cells Accumulate in the Heart After MI and Promote Healing**

Testing the 2 concepts of macrophage accumulation in MI required determining whether Nr4a1-expressing myeloid cells infiltrate the infarcted myocardium. First, immunohistochemistry showed enrichment of Nr4a1 in the infarcted area on days 3 and 7 after permanent ligation of the left anterior descending coronary artery when compared with sham controls (Figure 1A). Second, immunofluorescence revealed that myeloid CD11b+ cells dominated the infiltrate in the infarct and expressed Nr4a1 in the nucleus (Figure 1B). Other cells,
including endothelial cells and T cells, which were relatively rare, also stained for Nr4a1 (Online Figure I).

Next, we asked whether the receptor is functionally important in the myocardial infiltrate. We generated chimeric mice that lacked Nr4a1 in bone-marrow–derived, hematopoietic cells, including cardiac macrophages (for simplicity we call these mice Nr4a1–/–; Online Figure IIA–IIC). The approach allowed us to test the role of Nr4a1 in hematopoietic cells, while controlling for Nr4a1 in stromal cells, such as cardiomyocytes, fibroblasts, or endothelial cells. After reconstitution, we permanently ligated the left anterior descending coronary artery of these mice, along with controls (WT), and evaluated the healing response 7 days later. When compared with WT controls, the myocardium of Nr4a1–/– mice accumulated surprisingly more myeloid CD11b+ cells but had smaller regions of extracellular matrix deposition, reflected by collagen I, fewer reparative smooth muscle actin+ myofibroblasts, and less neovascularization, as determined by staining for CD31+ endothelial cells (Figure 2). Collectively, such a phenotype points to a defect in the healing process, suggesting that Nr4a1 deficiency retards the resolution of inflammation during MI.

Biphasic Ly-6C<sup>high</sup> Monocyte and Ly-6C<sup>low</sup> Macrophage Response After MI

The Mo/MΦ response after MI is biphasic. In the first phase, inflammatory Ly-6C<sup>high</sup> Mo/MΦ accumulate and participate in inflammation. In the second phase, reparative Ly-6C<sup>low</sup> Mo/MΦ contribute to collagen deposition and scar formation. The 2 phases may arise either via the sequential recruitment of circulating monocyte subsets or via local macrophage differentiation and polarization. The recent observations that Ly-6C<sup>low</sup> monocytes do not differentiate to macrophages, as was previously thought, but patrol vessels and mark endothelial cells for elimination argue against the sequential recruitment model and necessitate its re-evaluation. We focused on Nr4a1 because Nr4a1–/– mice lack Ly-6C<sup>low</sup> monocytes in the blood but can generate Ly-6C<sup>low</sup> macrophages in tissue. To track cell influx and differentiation, we developed a 9-color flow cytometry method (an improvement over the 4-color method in the original description of the biphasic response). We profiled the leukocyte population in the myocardium in the steady state and 3 and 7 days after coronary artery ligation, in both WT and Nr4a1–/– mice. In WT animals, we observed a peak of Ly-6C<sup>high</sup> monocytes on day 3 that waned by day 7 and a surge of Ly-6C<sup>low</sup> cells that peaked at day 7 (Figure 3A). This finding agrees with our previous observation. Nr4a1–/– mice, however, showed an early peak of Ly-6C<sup>high</sup> monocytes and, surprisingly, a late peak of Ly-6C<sup>low</sup> macrophages although these mice did not have Ly-6C<sup>low</sup> monocytes in the heart (Figure 3A) or in the blood (Figure 3B). Moreover, Nr4a1–/– mice had more, not less, monocytes and macrophages at the 2 time points (Figure 3C), which agrees with our histological data (Figure 2). Neutrophil infiltration peaked in MI tissue during the first 3 days, completely regressed thereafter, and was more prominent in Nr4a1–/– mice, whereas lymphocytes accumulated similarly in both groups (Online Figure III). Given the revised and stringent gating strategy, which discriminates between monocytes and macrophages by the expression of F4/80, CD68, and major histocompatibility complex (MHC) II, the data also indicate that the early Ly-6C<sup>high</sup> monocyte peak consists predominantly of Ly-6C<sup>high</sup> macrophages that have recently infiltrated the myocardium but have not yet differentiated to F4/80<sup>high</sup>, CD68<sup>high</sup>, and MHCII<sup>+</sup> macrophages. In contrast, the Ly-6C<sup>low</sup> cell peak consists predominantly of mature F4/80<sup>high</sup>, CD68<sup>high</sup>, and MHCII<sup>+</sup> macrophages. Ly-6C<sup>low</sup> monocytes infiltrated the tissue at low numbers. Thus, although these data agree with
monocytes remained relatively unchanged between days 1 and 467. GFP+ mice (Figure 4A) to CD45.1+ WT mice that had undergone coronary artery ligation 3 days earlier (Figure 4B). We chose to inject monocytes on day 3 after MI because this time point corresponded to the monocyte peak in the infarcted myocardium of Nr4a1–/– mice (Figure 3D).

**Ly-6C<sup>hi</sup> Monocytes Give Rise to Both the Inflammatory and the Reparative Phase**

The data in Nr4a1–/– mice precluded the possibility that Ly-6C<sup>lo</sup> macrophages arise from Ly-6C<sup>hi</sup> monocytes in MI, and prompted us to identify the precursors of Ly-6C<sup>lo</sup> macrophages directly with a fate-mapping strategy in WT mice. We injected either Ly-6C<sup>hi</sup> or Ly-6C<sup>lo</sup> monocytes from CD45.2<sup>+</sup> GFP<sup>+</sup> mice (Figure 4A) to CD45.1<sup>+</sup> WT mice that had undergone coronary artery ligation 3 days earlier (Figure 4B). We chose to inject monocytes on day 3 after MI because this time point corresponded to the monocyte peak in the infarct for both subsets (Figure 3). We then euthanized the animals 1 day (ie, 4 days after MI onset) and 3 days (ie, 6 days after MI onset) later and tracked the fate of the GFP<sup>+</sup> cells. In the blood, we observed GFP<sup>+</sup> cells on days 1 and 3 in both the Ly-6C<sup>hi</sup> and the Ly-6C<sup>lo</sup> groups (Figure 4C). Ly-6C<sup>lo</sup> monocytes remained relatively unchanged between days 1 and 3, insofar as they continued to be CD115<sup>+</sup> Ly-6C<sup>lo</sup>. However, Ly-6C<sup>hi</sup> monocytes were mostly CD115<sup>+</sup> Ly-6C<sup>lo</sup> on day 1 but entirely CD115<sup>+</sup> Ly-6C<sup>hi</sup> by day 3. These data agree with the long-held assertion that Ly-6C<sup>hi</sup> monocytes can convert to Ly-6C<sup>lo</sup> monocytes.24,25

We then evaluated the identity of GFP<sup>+</sup> cells in the infarct. As in the blood, we detected GFP<sup>+</sup> cells in both groups at both times. There were crucial differences, however, between the groups injected with Ly-6C<sup>hi</sup> versus Ly-6C<sup>lo</sup> monocytes. Already 1 day after MI, ≈50% of the accumulated Ly-6C<sup>hi</sup> monocytes augmented F4/80<sup>+</sup>. By day 3 (ie, day 6 after MI, which corresponds to phase 2), almost all of the Ly-6C<sup>hi</sup> monocytes had lowered Ly-6C and most of those became F4/80<sup>hi</sup> macrophages (ie, Ly-6C<sup>lo</sup> F4/80<sup>hi</sup> macrophages) (Figure 4D). In contrast, Ly-6C<sup>lo</sup> monocytes accumulated but did not increase F4/80 (Figure 4E). These data, as well as data generated using Nr4a1<sup>+/–</sup> mice, strongly argue that Ly-6C<sup>lo</sup> monocytes give rise to both the inflammatory Ly-6C<sup>hi</sup>–dominant and reparative Ly-6C<sup>lo</sup>–dominant Mo/MΦ accumulation phases.

**N4ra1 Deficiency Reduces Monocyte Recruitment into the Myocardium**

Having determined that Ly-6C<sup>hi</sup> monocytes stem both the inflammatory and the reparative phases in the infarcted myocardium, and having observed that in the absence of Nr4a1 the myocardium accumulates higher numbers of monocytes, we returned our attention to the role of Nr4a1, a receptor that has been implicated in many processes involved in cell proliferation and differentiation. We found major fluctuations of Nr4a1 expression in monocytes and macrophages sorted from the blood and the heart during infarct healing (Figure 4F). Considering the results of the previous fate-mapping experiment, our data show that Nr4a1<sup>hi</sup> Ly-6C<sup>hi</sup> monocytes that infiltrate during the inflammatory phase give rise to Nr4a1<sup>hi</sup> macrophages that accumulate in the infarcted tissue during the reparative phase. Macrophages at a distant site, such as the peritoneal cavity, showed low Nr4a1 expression without modulation (Figure 4G), emphasizing the local nature of the response. Importantly, augmented expression of Nr4a1 by cardiac macrophages in the second phase is associated with the rise of interleukin-10, tissue growth factor-β, and vascular endothelial growth factor (Figure 4G), which are key mediators of the reparative phase.3 These data suggest a functional role for Nr4a1 in the cells that participate in MI healing. Additional studies will need to determine how Nr4a1 is regulated. Although our data argue that regulation depends on the local environment, the precise mechanism responsible for Nr4a1 fluctuations may need additional study and may involve toll-like receptor and NF-xB–dependent signaling.26

We hypothesized that the increased monocyte number in the infarcted myocardium of Nr4a1<sup>+/–</sup> mice reflects either heightened monocyte production or heightened monocyte recruitment. Several studies have shown that monocytes infiltrating the myocardium are derived from the bone marrow and a splenic reservoir through medullary and extramedullary hematopoiesis.8–10,27 Yet, we found no significant differences in the number of circulating Ly-6C<sup>hi</sup> monocytes after MI, despite somewhat lower counts in the bone marrow of Nr4a1<sup>+/–</sup> mice (Figure 5A; Online Figure IV), suggesting that
monocyte production does not account for the differences in the infarct. To evaluate recruitment, we first observed that monocytes from *Nr4a1−/−* mice had a higher surface expression of the chemokine receptor, C–C chemokine receptor 2 (Ccr2; Figure 5B), which fluctuated and peaked on day 3 (Figure 5C). Ccr2 is critical to the mobilization of Ly-6C<sup>hi</sup> monocytes out of the bone marrow and their accumulation in the infarct. The observation that *Nr4a1−/−* Ly-6C<sup>hi</sup> monocytes expressed abnormally high levels of Ccr2 on their surface thus argued for monocyte mobilization and recruitment as a major mechanism. Moreover, coinciding with the surge of Ccr2 expression in *Nr4a1−/−* mice, we observed a peak of serum C–C chemokine ligand 2 (also known as monocyte chemotactic protein 1 [MCP-1]), the ligand for Ccr2, in both chimeric groups on day 3 (Figure 5D). We detected relatively minor differences between WT and *Nr4a1−/−* Ly-6C<sup>hi</sup> monocyte surface expression of other adhesion molecules implicated in monocyte recruitment, such as CD62L, P-selectin glycoprotein ligand-1, or macrophage antigen 1 (Figure 5E). These data imply that mobilization and recruitment, rather than production, accounted for differences in monocyte infiltration. Indeed, in vivo antagonism of Ccr2 reduced the number of Ly-6C<sup>hi</sup> monocytes in the blood and infarct on day 3 and eliminated the differences between the WT and *Nr4a1−/−* mice (Figure 5F). Thus, Nr4a1 limits monocyte influx by limiting the expression of Ccr2 on Ly-6C<sup>hi</sup> monocytes during the inflammatory phase of infarct healing.

Figure 3. Enhanced accumulation of *Nr4a1−/−* monocytes and macrophages in myocardial infarct tissue. A, Representative images for flow cytometric analysis of myocardial infarction (MI) tissue cell suspensions before and 3 and 7 days after permanent left anterior descending coronary artery ligation in wild-type (WT) and *Nr4a1−/−* mice. B, Flow cytometry and analogous gating strategy identifying blood monocyte subsets in WT and *Nr4a1−/−* chimeras at steady state. C, Flow cytometry–based quantification of monocyte and macrophage numbers in MI tissue of WT vs *Nr4a1−/−* mice before and 1, 3, 7, and 21 days after MI. Results are presented as mean±SEM, *P*≤0.05, n≥4 per group and time point. D, Graphs illustrating flux of monocyte and macrophage subsets during phase 1 (≤4 days) and phase 2 (days 4–21) of infarct healing. APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; Ly-6C, lymphocyte antigen 6C; MHC, major histocompatibility complex; Nr4a1, nuclear receptor subfamily 4, group a, member 1; and SSC, side scatter.
Our data show that Nr4a1 limits monocyte influx by limiting Ccr2 expression. Is Nr4a1 important in differentiated, Nr4a1* high, cardiac macrophages (Figure 4F)? Given the reported functions of Nr4a1 in other cells, we first focused on macrophage proliferation and survival. To study proliferation, we used Ki-67, a marker whose nuclear expression identifies cells...
Figure 5. Nuclear receptor subfamily 4, group a, member 1 (Nr4a1)-deficiency promotes C–C chemokine receptor (CCR2)-mediated monocyte recruitment to the myocardial infarct tissue. A, Quantification of monocyte subsets and neutrophils in the blood of wild-type (WT) and Nr4a1−/− mice before and on days 1, 3, 7, and 21 after myocardial infarction (MI). Results are presented as means±SEM, *P<0.05, n=25 per group and time point. B, Representative dot plot of flow cytometric staining for CCR2 expression on circulating monocytes in WT and Nr4a1−/− mice 3 days after MI. C, Quantification of CCR2 expression on Ly-6C+ monocytes by mean fluorescence intensity at indicated time points after MI. D, Quantification of serum monocyte chemotactic protein 1 (MCP-1)/C–C chemokine ligand (CCL2) levels in WT and Nr4a1−/− mice before and on days 1, 3, 7, and 21 after MI. Results are presented as means±SEM, n=25 per group and time point. E, Quantification of expression of L-selectin (CD62L), P-selectin glycoprotein ligand-1 (PSGL-1), and integrin Mac1 on Ly-6Chigh monocytes by mean fluorescence intensity at indicated time points after MI. F, Quantification of Ly-6C+ monocyte numbers in peripheral blood and MI tissue on day 3 after permanent left anterior descending coronary artery (LAD) ligation in WT and Nr4a1−/− mice treated with CCR2 antagonist (RS504393, 2 mg/kg ip BID) or vehicle (30% DMSO ip) alone. Results are presented as mean±SEM, *P<0.05, n=5 per group and time point.

in mitosis, and bromodeoxyuridine, a nucleotide analogue that, if delivered as a short pulse, identifies locally proliferating cells.25,31,32 We tested WT and Nr4a1−/− mice 7 days after MI, which corresponded to the macrophage peak. Remarkably, we found abundant Ki-67+ cells colocalized with CD11b in the myocardium (Figure 6A), indicating local proliferation of myeloid cells. To identify the cells more precisely, we used a flow cytometry approach with a bromodeoxyuridine pulse. Two hours after injection of bromodeoxyuridine, when monocytes in the blood (Figure 6B) and myocardium were still bromodeoxyuridine−, already a substantial fraction (ie, ≈3%, which is in the range reported for lesional macrophage proliferation after such a pulse)31 of mature F4/80+ macrophages in the myocardium were bromodeoxyuridine+ (Figure 6C), indicating local proliferation. Recent studies have identified proliferation as a major mechanism by which macrophages replenish themselves in infection and atherosclerosis.31,33 We now show that monocyte-derived cardiac macrophages also proliferate after MI. Cardiac macrophage proliferation, however, did not depend on Nr4a1 because the rate of proliferation between the 2 groups did not differ, as determined with either Ki67 (Figure 6A) or bromodeoxyuridine (Figure 6C).

To profile survival, we performed TdT-mediated dUTP nick-end labeling assays on tissue sections and measured expression of caspase-3 on cardiac monocytes and macrophages in WT and Nr4a1−/− mice. The TdT-mediated dUTP nick-end labeling assays identified dying CD11b+ cells, with no differences between the groups (Figure 6D). Flow cytometric analysis of apoptosis using caspase-3 expression supported our TdT-mediated dUTP nick-end labeling assay data and showed that ≈7% of monocytes and ≈3% of macrophages in both WT and Nr4a1−/− mice were dying (Figure 6E). Together, these data suggest that cardiac macrophages proliferate and die in the infarcted myocardium independently of Nr4a1.

Aside from proliferation and survival, Nr4a1 has also been implicated in controlling expression of various inflammatory genes. When compared with WT controls, cardiac macrophages sorted from Nr4a1−/− mice exhibited a more inflammatory signature, as defined by the transcription of Il1β, Il6, Il10, Tgfβ, Il12, Mmp2, Mmp3, Mmp9, Vegfa, Msr1, Tgfb3, Arg1, Il10, Ptgds, and CsF (Figure 6F). The data agree with the observation that Nr4a1 limits inflammation during the reparative phase (Figures 2 and 3).

Nr4a1 Protects Against LV Remodeling and Dysfunction

Would Nr4a1-dependent attenuation of cardiac inflammation and promotion of healing translate into better heart function? To test this hypothesis, we performed serial echocardiograms on WT and Nr4a1−/− mice before, and at 2 and 21 days after MI. We tracked individual changes in LV volume, ejection fraction, and fractional shortening during infarct healing and cardiac remodeling. As expected, within 2 days after permanent left anterior descending coronary artery ligation, acute myocardial ischemia reduced the ejection fraction similarly in both groups, thereby excluding a potential bias by differences in surgery between the groups. By day 21 after coronary artery ligation, however, Nr4a1−/− mice developed more severe LV...
dysfunction than WT mice, judged by indices such as ejection fraction, fractional shortening, and LV volumes (Figure 7A and 7B; Online Table I), findings that agree with an increased myocardial scar size with reduced collagen density in Nr4a1–/– mice (Figure 7C and 7D). Therefore, Nr4a1 attenuates impaired LV function and adverse cardiac remodeling via its actions on monocytes and macrophages in both inflammatory and reparative phases of the infarcted myocardium (Figure 8).

**Discussion**

Nr4a1 is emerging as an important regulator of gene expression in macrophages, but consensus on its function in cardiovascular disease is still lacking. An early report showed that Nr4a1 activates genes involved in inflammation, apoptosis, and cell cycle control. In murine atherosclerosis, the absence of Nr4a1 aggravated atherosclerosis, whereas its overexpression in lesional macrophages reduced inflammatory cytokine production. The hypothesis that Nr4a1 is predominantly anti-inflammatory is also consistent with arterial injury and restenosis studies, concluding that the receptor inhibits vascular outward remodeling and reduces macrophage accumulation. Recent challenges on the importance of Nr4a1 in atherosclerosis, however, have reignited interest in the receptor’s range of influence.

![Figure 6](image_url)
In this study, we have shown that the inflammatory response during MI is heightened, healing is compromised, and indices of heart failure worsen in mice lacking expression of Nr4a1 on hematopoietic cells. The events that influence macrophage accumulation and function in response to injury involve monocyte production in the bone marrow, monocyte mobilization from the bone marrow and a splenic reservoir, influx of monocytes into tissue, and differentiation to macrophages. Once differentiated, macrophages influence their environment through production of various mediators, including cytokines, proteases, oxidases, and scavenger receptors. Eventually macrophages die in situ. This study confirmed that Nr4a1 contributes not only to Ly-6C<sup>low</sup> but also to Ly-6C<sup>high</sup> monocyte numbers in the bone marrow. In response to MI, we found that Nr4a1-deficient Ly-6C<sup>high</sup> monocytes express high surface levels of Ccr2, which augments cell mobilization and influx, possibly explaining why, despite somewhat lower numbers in the steady state, monocyte infiltration rises when compared with WT. Once differentiated, cardiac macrophages rely on Nr4a1 to limit inflammation, but Nr4a1 does not seem to regulate apoptosis or proliferation. Thus, Nr4a1 limits both the influx of inflammatory monocytes to the myocardium and the expression of inflammatory mediators by monocyte-derived macrophages. Future studies will need to determine whether those processes are linked and how Nr4a1 influences other cells in MI, such as cardiomyocytes, endothelial cells, and T cells. It will also be important to discern whether additional mechanisms, perhaps mediated by neutrophils, contribute to the heightened influx of monocytes.

In addition to providing novel insights into the role of Nr4a1 during MI, this study also revisited the biphasic model of macrophage accumulation, originally described by us...
and interpreted as evidence for sequential monocyte subset recruitment. We used Nr4a1, which is essential to Ly-6C<sub>low</sub> monocytes but dispensable to Ly-6C<sub>low</sub> macrophages, as a tool to discriminate between sequential recruitment and local macrophage differentiation. Combining advanced flow cytometry and fate-mapping approaches, we show that the biphasic response originates from Ly-6C<sub>high</sub> monocytes, which accumulate in the infarct early and, over time, as they differentiate to macrophages, lose Ly-6C expression. Although this revised model agrees with the initial description of biphasic macrophage accumulation, it departs from it in 1 critical interpretation: the first Ly-6C<sub>high</sub> monocyte-dominant phase is followed not by a Ly-6C<sub>low</sub> monocyte but by a Ly-6C<sub>high</sub> monocyte-derived Ly-6C<sub>low</sub> macrophage-dominant phase. This expanded concept highlights the importance of the local environment in orchestrating inflammation and healing.

Macrophage proliferation, which has garnered considerable attention recently, 33,44,45 is an example of a process that depends on the local environment. Our own observations in experimental atherosclerosis have revealed that proliferation of monocyte-derived macrophages dominates accumulation of lesional macrophages in established disease. 31 Here, we build on these and other observations 46 by providing evidence that monocyte-derived cardiac macrophages proliferate robustly—and independently of Nr4a1—in the infarcted myocardium. The data suggest that after initial Ly-6C<sub>high</sub> monocyte recruitment and differentiation, macrophages can also replenish themselves locally. Future studies will, therefore, need to determine the relative contribution and relevance of macrophage proliferation over the course of healing after MI. Our enumeration of Ly-6C<sub>high</sub> and Ly-6C<sub>low</sub> monocytes in the myocardium for the first 7 days after MI, combined with data on Ccr2 and C–C chemokine ligand 2 expression, supports the concept that the first phase requires intense Ly-6C<sub>high</sub> monocyte infiltration that sharply drops in the second phase.

In light of these findings, the role of Ly-6C<sub>low</sub> monocytes in MI remains uncertain. Ly-6C<sub>low</sub> monocytes might nevertheless contribute indispensably (eg, by patrolling the vasculature and marking damaged endothelial cells for disposal). 21–23

MI and ischemic cardiomyopathy are major causes of morbidity and mortality worldwide. Decades of work explored the role of cardiomyocytes, cardiac fibroblasts, stem cells, and the extracellular matrix 47–50 in heart function and its failure after MI. Macrophages, which have received much less attention in this context, are emerging as key protagonists that can either promote inflammation or contribute to its resolution. This study has shed new mechanistic light onto monocyte and macrophage functions during MI by showing that the nuclear hormone receptor, Nr4a1, modulates both the early Ly-6C<sub>high</sub> monocyte-dominant inflammatory and the later Ly-6C<sub>low</sub> macrophage-dominant reparative phases in the infarcted myocardium.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Transcription factor nuclear receptor subfamily 4, group a, member 1 (Nr4a1) is essential to lymphocyte antigen 6C (Ly-6C)low but not to Ly-6Chigh monocyte development.
- Accumulation of monocytes and macrophages in the infarcted myocardium is biphasic, involving an early Ly-6Chigh inflammatory phase and a later Ly-6Clow reparative phase.
- The inflammatory response is exaggerated in lipopolysaccharide-stimulated Nr4a1-deficient bone marrow–derived macrophages.

**What New Information Does This Article Contribute?**

- Ly-6Chigh monocytes give rise to both the Ly-6Chigh inflammatory and the Ly-6Clow reparative phases.
- Nr4a1 expression limits C–C chemokine receptor 2–mediated recruitment of Ly-6Chigh monocytes to the infarcting tissue.
- Ly-6Chigh macrophages derive from Ly-6Chigh monocytes, proliferate and use Nr4a1 to dampen inflammation.

Myocardial infarction involves accumulation of monocytes and macrophages in tissue. Monocytes in mice comprise ≥2 functionally distinct subsets, exhibiting different expression of Ly-6C. The orphan nuclear receptor Nr4a1 is essential to the development of Ly-6Clow but not of Ly-6Chigh monocytes. We, therefore, used Nr4a1-deficient mice to study how Ly-6Clow monocytes and Nr4a1 expression in cardiac macrophages contribute to myocardial infarct healing. Ly-6Clow monocytes accumulated at low numbers in the infarct tissue and did not differentiate into cardiac macrophages. In contrast, inflammatory Ly-6Chigh monocytes dominated the infiltrate within the first 3 days after induction of myocardial infarction, differentiated into Ly-6Chigh macrophages, dominated the second healing phase, and proliferated. This process coincided with elevated Nr4a1 expression in cardiac macrophages and increased the expression of regenerative factors. Nr4a1 expression, albeit lower in Ly-6Chigh monocytes, corresponded with suppressed expression of chemokine receptor, C–C chemokine receptor 2, limiting recruitment of inflammatory cells to the infarct. Consequently, Nr4a1 deficiency resulted in adverse cardiac remodeling during myocardial infarct healing and impaired cardiac function. Nr4a1 thus regulates the coordinated biphasic monocyte/macrophage response during myocardial infarction.
Supplemental Methods

Animal models and in vivo interventions. Bone marrow chimeras: 8 weeks old female C57BL/6J (wild-type[wild-type]) mice were lethally irradiated (950cGy) and reconstituted with WT (Controls) and Nr4a1–/– bone marrow cells, respectively, for 10 weeks. Model of myocardial infarction: Myocardial infarction (MI) was induced by permanent ligation of the left anterior descending coronary artery (LAD). Mice were anesthetized with isoflurane (2%/2 liters O2), intubated and ventilated with an Inspira Advanced Safety Single Animal Pressure/Volume Controlled Ventilator (Harvard Apparatus). Left thoracotomy was performed in the forth intercostal space after shaving the chest wall. The left ventricle was visualized and the LAD was ligated with monofilament 8-0 suture (Ethicon, Somerville, NJ). The chest and skin were closed with a 7-0 nylon suture followed by removal of air from the thorax via a pleural catheter. The procedure was performed by the same surgeon blinded to genotypes. Adoptive transfer: Monocytes from C57BL/6-Tg(UBC-GFP)30Scha/J mice were enriched by magnetic cell separation prior to flow assisted cell sorting. Pooled cell suspensions from spleens and blood were incubated with 4 µl anti-CD11b-APC-Cy7 Ab (Biolegend, San Diego, CA, USA) per 1 x 10^8 cells in sterile 2% FBS (fetal bovine serum, Atlanta Biologicals, Lawrenceville, GA, USA), 0.5% BSA (bovine serum albumin, MP Biomedicals, Solon, OH, USA) in PBS for 30 min on ice, washed and incubated with 100 µl anti-APC MACS beads (Miltenyi Biotec, Auburn, CA, USA) per 1 x 10^8 cells in 0.5% BSA, 2mM EDTA in PBS for another 30 min on ice. Labeled cells were positively selected in a Midi MACS separator and LS column according to the manufacturer’s instructions and then stained for anti-Lin-PE (Lin (lineage) = Ter119, CD3, CD90.2, CD19, B220, NK1.1, CD49b, Ly6G), anti-MHCII-PerCp-Cy5.5, anti-F4/80-PECy7, anti-CD115-APC, anti-Ly6C-Pacific Blue and anti-CD45.2-Pacific Blue. CD45.2+ and CD45.2- monocytes were sorted separately on a FACS Aria II cell sorter (BD Biosciences). At 3 days post MI CD45.1+ WT mice received either 1 x 10^6 GFP+ Ly6C^high or 0.5 x 10^6 GFP+ Ly6C^low monocytes /mouse by tail vein injection. Mice were sacrificed 1 and 3 days post transfer, respectively. CCR2 antagonist: Mice were intraperitoneally (i.p.) injected with 2mg/kg RS504393 or vehicle (30% DMSO) twice daily (b.i.d) from day 1 to day 3 post MI and sacrificed 3 hours post last injection.

Cell isolation. Peripheral blood was collected by retroorbital bleeding with heparinized capillaries, and erythrocytes were lysed in RBC Lysis buffer (Biolegend). Peritoneal lavages and organs were harvested at the day of sacrifice. Femurs and hearts were excised after vascular perfusion with 10 ml sterile PBS. Flushed bone marrow was strained through a 40 µm-nylon mesh (BD Biosciences, San Jose, CA, USA). The healthy left ventricle or infarcted areas were excised including the border zone, minced and digested in 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I and 60 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) PBS for 1 h at 37°C while shaking. Cells were counted in a Neubauer chamber. One femur contains ~5% of all bone marrow cells. Bone marrow cell counts were extrapolated accordingly. Cell sorting: Heart tissues from C57BL/6J mice, Nr4a1–/– and WT bone marrow chimeras were minced and digested as described above and stained for anti-Ly6C-Fitc, anti-Lin-PE (Lin (lineage) = Ter119, CD3, CD90.2, CD19, B220, NK1.1, CD49b, Ly6G), anti-MHCII-PerCp-Cy5.5, anti-F4/80-PECy7, anti-CD68-APC, anti-CD11c-Alexa Fluor 700, anti-CD11b-APCCy7, anti-CD45.2-Pacific Blue and anti-CD115-Streptavidin Pacific Orange. Blood was lysed prior to staining with equivalent antibodies. Monocyte subsets and macrophages were identified as shown in Figure 3. Peritoneal macrophages were identified in peritoneal lavage as CD45.2+ SSC^high CD11b+ F4/80^high cells. Cells were sorted on a FACS Aria II cell sorter (BD Biosciences) directly into RLT buffer for subsequent RNA isolation.

Serum analysis. Serum was collected from retroorbital blood before and at 1, 3, 7 and 21 days post MI. CCL2/MCP-1 serum levels were measured with the Mouse CCL2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.
**Histology.** Hearts were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), frozen in ice-cold 2-Methylbutane (Fisher Scientific, Fair Lawn, NJ) and sectioned into 6 µm slices yielding 30-40 sections per mouse. The following antibodies were used for immunohistology: Anti-Nur77 (clone M-210, Santa Cruz Biotechnology, Inc.), anti-CD11b (clone M1/70, BD Biosciences), anti-Collagen I (ab21286, Abcam), anti-CD31 (clone MEC13.3, BD Biosciences), anti-α-smooth muscle actin (ab5694; Abcam) for smooth muscle cells (SMA). Biotinylated secondary antibodies followed by VECTASTAIN ABC reagent (Vector Laboratories, Inc. Burlingame, CA) were applied and the color development was performed using AEC substrate (Dako North America, Inc. Carpinteria, CA). Masson’s Trichrome staining was performed in cross sections of paraffin-embedded hearts 21 days post MI. For immunofluorescence staining anti-Ki67-FITC (clone SP6, Abcam), anti-CD11b (clone M1/70, BD Biosciences), anti-CD31 (clone MEC 13.3, BD Biosciences), secondary biotinylated anti-rat IgG and streptavidin DyLight 594 (Vector Laboratories), and Vectashield mounting medium with DAPI (Vector Laboratories) were used. TUNEL staining was carried out using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacture’s instructions. Images capture was performed using a Nanozoomer 2.0RS (Hamamatsu, Japan) and Olympus BX63 (Olympus America Inc., Center Valley, PA) equipped with an ANDOR Neo sCMOS Monochrome Camera (ANDOR technology, Northern Ireland). Images were analyzed with ImageJ.

**Flow Cytometry.** Cell suspensions were stained in PBS supplemented with sterile 2% FBS and 0.5% BSA. The following monoclonal antibodies were used for flow cytometric analysis: anti-Ly6C (clone AL-21, BD Biosciences), anti-CD45.1 (clone A20, Biolegend), anti-CD45.2 (clone 104, BD Biosciences), anti-CD3e (clone 145-2C11, ebioscience), anti-CD90.2 (clone 53-2.1, BD Biosciences), anti-CD19 (clone 6D5, Biolegend), anti-B220 (clone RA3-6B2, BD Biosciences), anti-MHCII (clone AF6-120.1, BD Biosciences), anti-F4/80 (clone BM8, Biolegend), anti-CD49b (clone DX5, BD Biosciences), anti-NK1.1 (clone PK136, BD Biosciences), anti-CD11b (clone M1/70, BD Biosciences), anti-CD11c (clone HL3, BD Biosciences), anti-CD68 (clone FA-11, Biolegend), and isotype controls. For intracellular staining cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions. Viable cells were identified as unstained with Zombie Yellow (Biolegend). Data were acquired on a LSRII and analyzed with FlowJo (Tree Star, Ashland, OR, USA). Specifically, monocytes were identified as CD45⁺, Lin⁻ (Lin = Ter119, CD3, CD90.2, CD19, B220, NK1.1, CD49b, Ly6G), CD11b⁺, F4/80low, MHCII⁺low, CD11c⁺low, CD68low, CD115⁺ cells, subdivided into Ly6Chigh and Ly6Clow subsets. Neutrophils were identified as CD45⁺, Lin⁺, CD11b⁺, MHCII⁺low, CD11c⁺low, SSChigh Ly6Cint cells. Cardiac macrophages were identified as CD45⁺, Lin⁻ (Lin = Ter119, CD3, CD90.2, CD19, B220, NK1.1, CD49b, Ly6G), CD11b⁺, F4/80high (CD68high, MHCII⁺) cells.

**Real-time PCR.** 2-4 x 10⁴ sorted monocytes and macrophages from blood, heart and peritoneum, respectively, were lysed in RLT buffer. RNA was isolated using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands) followed by cDNA transcription with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers’ instructions. Quantitative real-time TaqMan PCR was performed using the following TaqMan gene expression assays (Applied Biosystems): IL-1β (Mm01336189_m1), IL-6 (Mm00446190_m1), IL-12 (Mm00434165_m1), TNFα (Mm00443260_g1), TLR2 (Mm01213946_g1), TLR4 (Mm00445273_m1), TLR9 (Mm00446193_m1), MMP2 (Mm00439498_m1), MMP3 (Mm00440295_m1), MMP9 (Mm00442991_m1), VEGFα (Mm01281449_m1), Msr-1 (Mm00446214_m1), CD36 (Mm01135198_m1), IL-10 (Mm00439614_m1), TGFβ1 (Mm01178820_m1), Fizz (Mm00445109_m1), Arg1 (Mm00475988_m1), CD206 (Mm00485148_m1), Nr4a1 (Mm01300401_m1) and housekeeping gene β-actin (4352341E). PCR was run on a 7500 PCR thermal cycler (Applied Biosystems). Data were quantified with the 2⁻ΔCt method.
Echocardiography: Serial closed chest transthoracic echocardiography was performed under light anesthesia (ketamine 80 mg/kg ip) at baseline, 2 and 21 days post myocardial infarction. Images were acquired with a 13-MHz linear-array transducer (Vivid 7, GE Medical Systems). All measurements were performed offline on EchoPacs (GE Medical System) and each measurement reported was an average of 9 cardiac cycles. Left ventricular end-diastolic (LVID\textsubscript{ED}) and end-systolic diameters (LVID\textsubscript{ES}) were obtained from M-mode tracings from 2D parasternal short-axis views at the mid and apical levels at each time point\textsuperscript{1}. Fractional shortening (FS) was calculated as $\text{FS} = (\text{LVID}_{\text{ED}} - \text{LVID}_{\text{ES}})/\text{LVID}_{\text{ED}}$. LV end-diastolic and systolic volumes (LVEDV and LVESV) were derived using measurements performed in the parasternal long axis 2 dimensional view and the area-length method\textsuperscript{2}. Left ventricular ejection fraction (LVEF) was calculated as $\text{LVEF} = (\text{LVEDV} - \text{LVESV})/\text{LVEDV} \times 100\%$.

Online Figures

Online Figure I: Immunofluorescence co-staining for Nr4a1 with CD31 and CD3 in MI tissue 7 days after coronary artery ligation. Representative images from one out of three samples are shown.
Online Figure II: A. Scheme illustrating the generation of \( Nr4a1^{-/-} \) and wild type (WT) bone marrow chimeras in C57Bl/6 mice. B. Evaluation of the replacement of cardiac macrophages after lethal irradiation and reconstitution of a CD45.2\(^+\) recipient with CD45.1\(^+\) donor bone marrow before and after myocardial infarction (MI). C. Quantification of \( Nr4a1 \) expression in macrophages sorted from MI tissue on day 7 after permanent LCA ligation. Results are presented as mean ± SEM, \( n \geq 3 \) per group. n.d.= non-detectable.

Online Figure III: Flow cytometry-based quantification of neutrophil (Lin\(^+\) CD11b\(^+\)) and Lin\(^+\) CD11b\(^-\) lymphocyte (T, B and Natural killer cells) numbers in MI tissue of WT versus \( Nr4a1^{-/-} \) mice before and 1, 3, 7, and 21 days post MI. Results are presented as mean ± SEM, \(* p \leq 0.05\), \( n \geq 4 \) per group and time point.
Online Figure IV: Quantification of Ly-6C<sup>high</sup> monocytes and neutrophils in the bone marrow of WT and Nr4a1<sup>−/−</sup> mice before and on days 1, 3, 7 and 21 post MI. Results are presented as mean ± SEM, * p ≤ 0.05, n ≥5 per group and time point.
### Online Table I: Heart function parameters assessed by echocardiography before, 2 and 21 days post MI in WT and Nr4a1−/− mice. Results are presented as mean ± SEM, *p ≤ 0.05, n =8 per group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>2 days post MI</th>
<th>21 days post MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Nr4a1−/−</td>
<td>WT</td>
</tr>
<tr>
<td>EF (%)</td>
<td>59.98 ± 0.75</td>
<td>59.66 ± 0.48</td>
<td>36.14 ± 0.69</td>
</tr>
<tr>
<td>EDV (mm³)</td>
<td>50.72 ± 0.68</td>
<td>49.93 ± 1.77</td>
<td>60.14 ± 0.72</td>
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<tr>
<td>ESV (mm³)</td>
<td>20.34 ± 0.53</td>
<td>20.09 ± 0.62</td>
<td>38.40 ± 0.24</td>
</tr>
<tr>
<td>Papillary FS (%)</td>
<td>43.11 ± 0.68</td>
<td>43.57 ± 0.43</td>
<td>27.00 ± 0.85</td>
</tr>
<tr>
<td>Apical FS (%)</td>
<td>59.56 ± 0.84</td>
<td>61.00 ± 0.82</td>
<td>12.00 ± 0.58</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>578 ± 16</td>
<td>592 ± 11</td>
<td>516 ± 14</td>
</tr>
</tbody>
</table>

### Supplemental References
