



Published in final edited form as:

Circ Res. 2014 May 9; 114(10): 1611–1622. doi:10.1161/CIRCRESAHA.114.303204.

Ly-6C^{high} Monocytes Depend on Nr4a1 to Balance both Inflammatory and Reparative Phases in the Infarcted Myocardium

Ingo Hilgendorf¹, Louisa M. S. Gerhardt¹, Timothy C. Tan², Carla Winter¹, Tobias A. W. Holderried³, Benjamin G. Chousterman¹, Yoshiko Iwamoto¹, Ronglih Liao⁴, Andreas Zirlik⁵, Marielle Scherer-Crosbie², Catherine C. Hedrick⁶, Peter Libby⁷, Matthias Nahrendorf¹, Ralph Weissleder^{1,8}, and Filip K. Swirski¹

¹Center for Systems Biology, Massachusetts General Hospital, Boston, MA, USA

²Department of Cardiology, Massachusetts General Hospital, Boston, MA, USA

³Department of Gastroenterology, Hepatology and Infectious Diseases, University of Duesseldorf, Duesseldorf, Germany

⁴Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

⁵Department of Cardiology and Angiology I, University Heart Center Freiburg, Freiburg, Germany

⁶La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

⁷Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

⁸Department of Systems Biology, Harvard Medical School, Boston, MA, USA

Abstract

Rationale—Healing after myocardial infarction (MI) involves the biphasic accumulation of inflammatory Ly-6C^{high} and reparative Ly-6C^{low} monocytes/macrophages (Mo/MΦ). According to one 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 one circulating subset via local macrophage differentiation and polarization. The orphan nuclear hormone receptor, Nr4a1, is essential to Ly-6C^{low} monocyte production but dispensable to Ly-6C^{low} 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 MI in the context of the two Mo/MΦ accumulation scenarios.

Address correspondence to: Dr. Filip K. Swirski, Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Simches Research Building, 185 Cambridge St., Boston, MA 02114, Tel: 617-724-6242, Fax: 617-726-5708, fswirski@mgh.harvard.edu.

I.H. and L.M.S.G contributed equally to this work.

DISCLOSURES

None.

Methods and Results—We show that Ly-6C^{high} monocytes infiltrate the infarcted myocardium and, unlike Ly-6C^{low} monocytes, differentiate to cardiac macrophages. In the early, inflammatory phase of acute myocardial ischemic injury, Ly-6C^{high} monocytes accrue in response to a brief Ccl2 burst. In the second, reparative phase, accumulated Ly-6C^{high} monocytes give rise to reparative Ly-6C^{low} F4/80^{high} macrophages that proliferate locally. In the absence of Nr4a1, Ly-6C^{high} monocytes express heightened levels of Ccr2 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^{high} monocytes orchestrate both inflammatory and reparative phases during MI and depend on Nr4a1 to limit their influx and inflammatory cytokine expression.

Keywords

Monocyte; macrophage; myocardial infarction; nuclear hormone receptor; healing

INTRODUCTION

Myocardial infarction (MI) is a leading cause of death worldwide^{1, 2}. Although the mortality rate from MI has declined steadily over the last 50 years due to 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 post-infarction decisively. Adverse remodeling of the infarcted left ventricle associates with the development of heart failure due to systolic dysfunction and ischemic mitral regurgitation, whereas avoidance 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 death³. On the other hand, macrophage-induced inflammation can be harmful and cause post-MI heart failure^{4, 5}. The molecular pathways that balance inflammatory and reparative macrophage functions therefore comprise novel potential targets of therapeutic intervention after MI.

Shortly after onset of ischemia, endothelial cells augment adhesion molecule expression that, along with released chemokines, trigger leukocyte mobilization and extravasation⁶. Within days, numerous leukocytes of medullary and extramedullary origins accumulate in the infarcting myocardium in massive numbers^{7–11}. Among them, phagocytic monocyte-derived macrophages digest dead tissue, efferocytose, influence collagen deposition, and promote angiogenesis. Optimal healing of the myocardium involves the biphasic accumulation of myeloid cells expressing the surface glycoprotein Ly-6C: inflammatory Ly-6C^{high} Mo/MΦ accumulate early, while 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 setting⁹, or whether the response evolves via local differentiation and polarization of macrophages, as has been shown in other situations^{12–15}. 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 Nr4a1 (also known as Nur77) has gained attention as a molecular switch that controls many cellular functions involved in inflammation, apoptosis, and proliferation^{16–19}. Recent studies have shown that Nr4a1 is essential to the development of Ly-6C^{low} monocytes²⁰, but is dispensable to the differentiation of Ly-6C^{low} macrophages¹⁵. 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 myocardial infarction occurs via the sequential differentiation of monocyte subsets or via the local differentiation of macrophages.

METHODS

For further details, see the online Supplemental Material.

Animals and animal experiments

Female C57BL/6J (WT), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1⁺) and C57BL/6-Tg(UBC-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, USA. 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. Myocardial infarction (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 post 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 online Supplemental Material. Data were acquired on a BD LSRII and analyzed with FlowJo. Cells were sorted with BD FACS AriaII.

Reverse transcription PCR

RNA was isolated from sorted cells with the RNeasy Micro Kit (Qiagen). Quantitative real-time TaqMan PCR was run on a 7500 PCR 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 (FS) was calculated using end-diastolic (ED) and end-systolic (ES) left ventricular inner diameters (LVID) as $FS = (LVID_{ED} - LVID_{ES})/LVID_{ED}$. Left ventricular ejection fraction (LVEF) was calculated using end-diastolic and end-systolic volumes as $LVEF = (LVEDV - LVESV)/LVEDV \times 100\%$.

Statistics

Results are shown as mean \pm SEM. The unpaired Student's t test was applied to evaluate differences between two study groups. One-way ANOVA with post-hoc Tukey's multiple comparisons test was performed when comparing more than two groups. P-values of 0.05 and less denote significant changes.

RESULTS

Nr4a1 expressing myeloid cells accumulate in the heart after MI and promote healing

Testing the two 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 (LAD) compared to 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-C). 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 LAD of these mice, along with controls (WT), and evaluated the healing response 7 days later. Compared to 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 (SMA)⁺ 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.

The biphasic Ly-6C^{high} monocyte and Ly-6C^{low} macrophage response after MI

The Mo/M Φ response after MI is biphasic⁹. In the first phase, inflammatory Ly-6C^{high} Mo/M Φ accumulate and participate in inflammation. In the second phase, reparative Ly-6C^{low} Mo/M Φ contribute to collagen deposition and scar formation. The two phases may arise either via the sequential recruitment of circulating monocyte subsets or via local

macrophage differentiation and polarization^{9, 12–15}. The recent observations that Ly-6C^{low} monocytes do not differentiate to macrophages, as was previously thought, but patrol vessels and mark endothelial cells for elimination^{21–23} argues against the sequential recruitment model and necessitates its re-evaluation. We focused on Nr4a1 because *Nr4a1*^{-/-} mice lack Ly-6C^{low} monocytes in the blood²⁰ but can generate Ly-6C^{low} macrophages in tissue¹⁵.

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 response⁹). 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^{high} monocytes on day 3 that waned by day 7, and a surge of Ly-6C^{low} cells that peaked at day 7 (Figure 3A). This finding agrees with our previous observation⁹. *Nr4a1*^{-/-} mice, however, showed an early peak of Ly-6C^{high} monocytes and, surprisingly, a late peak of Ly-6C^{low} macrophages, although these mice did not have Ly-6C^{low} monocytes in the heart (Figure 3A) or the blood (Figure 3B). Moreover, *Nr4a1*^{-/-} mice had more, not less, monocytes and macrophages at the two 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, while 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 MHCII, the data also indicate that the early Ly-6C^{high} monocyte peak consists predominantly of Ly-6C^{high} monocytes that have recently infiltrated the myocardium but have not yet differentiated to F4/80^{high} CD68^{high} MHCII⁺ macrophages. In contrast, the Ly-6C^{low} cell peak consists predominantly of mature F4/80^{high} CD68^{high} MHCII⁺ macrophages. Ly-6C^{low} monocytes infiltrated the tissue at low numbers. Thus, while these data agree with the original description of a biphasic Mo/MΦ response in MI, they argue against sequential recruitment and in favor of a Ly-6C^{high} monocyte-dominant phase 1 followed a Ly-6C^{low} macrophage-dominant phase 2 (Figure 3D).

Ly-6C^{high} monocytes give rise to both the inflammatory and reparative phase

The data in *Nr4a1*^{-/-} mice precluded the possibility that Ly-6C^{low} macrophages arise from Ly-6C^{low} monocytes in MI, and prompted us to identify the precursors of Ly-6C^{low} macrophages directly with a fate-mapping strategy in WT mice. We injected either Ly-6C^{high} or Ly-6C^{low} monocytes from CD45.2⁺ 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 post MI because this time-point corresponded to the monocyte peak in the infarct for both subsets (Figure 3). We then sacrificed the animals 1 day (i.e. 4 days after MI onset) and 3 days (i.e., 6 days after MI onset) later, and tracked the fate of the GFP⁺ cells. In the blood, we observed GFP⁺ cells on day 1 and day 3 in both the Ly-6C^{high} and Ly-6C^{low} groups (Figure 4C). Ly-6C^{low} monocytes remained relatively unchanged between day 1 and day 3, insofar as they continued to be CD115⁺ Ly-6C^{low}. However, Ly-6C^{high} monocytes were mostly CD115⁺ Ly-6C^{high} on day 1, but entirely CD115⁺ Ly-6C^{low} by day 3. These data agree with the long-held assertion that Ly-6C^{high} monocytes can convert to Ly-6C^{low} monocytes^{24, 25}.

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

Nr4a1 deficiency reduces monocyte recruitment into the myocardium

Having determined that Ly-6C^{high} monocytes stem both the inflammatory and 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^{low} Ly-6C^{high} monocytes that infiltrate during the inflammatory phase give rise to Nr4a1^{high} 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 associated with the rise of IL-10, TGFβ and VEGF (Figure 4G), which are key mediators of the reparative phase⁹. These data suggest a functional role for Nr4a1 in the cells that participate in MI healing. Future 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 need further study and may involve Toll-like receptor and NF-κB-dependent signaling²⁶.

We hypothesized that the increased monocyte number in the infarcted myocardium of *Nr4a1*^{-/-} mice reflects either heightened monocyte production or heightened monocyte recruitment. A number of studies have shown that monocytes infiltrating the myocardium derive 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 Ly6C^{high} monocytes after MI, despite somewhat lower counts in the bone marrow of *Nr4a1*^{-/-} 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 Ccr2 (Figure 5B), which fluctuated and peaked on day 3 (Figure 5C). Ccr2 is critical to the mobilization of Ly-6C^{high} monocytes out of the bone marrow and their accumulation in the infarct²⁸⁻³⁰. The observation that *Nr4a1*^{-/-} Ly-6C^{high} 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 Ccl2 (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^{high} monocyte surface expression of other adhesion molecules implicated in monocyte recruitment, such as CD62L, PSGL1, or Mac1 (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^{high} 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^{high} monocytes during the inflammatory phase of infarct healing.

Nr4a1-macrophages proliferate and are more inflammatory

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 utilized Ki-67, a marker whose nuclear expression identifies cells in mitosis, and BrdU, 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 co-localized 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 BrdU-pulse. 2 h after injection of BrdU, when monocytes in the blood (Figure 6B) and myocardium were still BrdU⁻, already a substantial fraction (i.e., ~3%, which is in the range reported for lesional macrophage proliferation after such a pulse³¹) of mature F4/80^{high} macrophages in the myocardium were BrdU⁺ (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 two groups did not differ, as determined with either Ki67 (Figure 6A) or BrdU (Figure 6C).

To profile survival, we performed TUNEL assays on tissue sections and measured expression of caspase-3 on cardiac monocytes and macrophages in WT and *Nr4a1*^{-/-} mice. The TUNEL assays identified dying CD11b⁺ cells, with no differences between the groups (Figure 6D). Flow cytometric analysis of apoptosis using caspase-3 expression supported our TUNEL 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. Compared to WT controls, cardiac macrophages sorted from *Nr4a1*^{-/-} mice exhibited a more inflammatory signature, as defined by the transcription of *Il1 β* , *Il6*, *Il2*, *Tnfa*, *Tlr2*, *Tlr4*, *Tlr9*, *Mmp2*, *Mmp3*, *Mmp9*, *Vegfa*, *Msr1*, *Tgfb*, *Il10*, *Fizz*, *Arg1*, and *CD206* (Figure 6F). The data agree with the observation that *Nr4a1* limits inflammation during the reparative phase (Figures 2 & 3).

Nr4a1 protects against left ventricular 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 left ventricular volume, ejection fraction, and fractional shortening during infarct healing and cardiac remodeling. As expected, within 2 days after permanent LAD 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 post coronary artery ligation, however, *Nr4a1*^{-/-} mice developed more severe left ventricular dysfunction than WT mice, judged by indices such as ejection fraction, fractional shortening, and left ventricular volumes (Figure 7A, B, Online Table I), findings that agree with an increased myocardial scar size with reduced collagen density in *Nr4a1*^{-/-} mice (Figure 7C, D). Therefore, *Nr4a1* attenuates impaired left ventricular 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 control¹⁸. In murine atherosclerosis, the absence of Nr4a1 aggravated atherosclerosis^{34, 35}, while its over-expression in lesional macrophages reduced inflammatory cytokine production³⁶. 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 accumulation³⁷. Recent challenges on the importance of Nr4a1 in atherosclerosis³⁸, however, have reignited interest in the receptor's range of influence.

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 receptors¹⁰. Eventually macrophages die in situ²⁷. This study confirmed that Nr4a1 contributes to Ly-6C^{low} – but also to Ly-6C^{high} – monocyte numbers in the bone marrow²⁰. In response to MI we found that Nr4a1-deficient Ly-6C^{high} 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 compared to wild type. Once differentiated, cardiac macrophages rely on Nr4a1 to limit inflammation, but Nr4a1 does not appear 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^{39–42}. It will also be important to discern whether additional mechanisms, perhaps mediated by neutrophils, contribute to the heightened influx of monocytes⁴³.

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^{low} monocytes but dispensable to Ly-6C^{low} 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^{high} 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 one critical interpretation: the first Ly-6C^{high} monocyte-dominant phase is followed not by a Ly-6C^{low} monocyte, but by a Ly-6C^{high} monocyte-derived Ly-6C^{low} 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 one 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 disease³¹. Here, we build on these and other observations⁴⁶ 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^{high} 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^{high} and Ly-6C^{low} monocytes in the myocardium over the first 7 days after MI, combined with data on Ccr2 and Ccl2 expression, supports the concept that the first phase requires intense Ly-6C^{high} monocyte infiltration that sharply drops in the second phase. In light of these findings, the role of Ly-6C^{low} monocytes in MI remains uncertain. Ly-6C^{low} monocytes might nevertheless contribute indispensably, for example 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 post 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^{high} monocyte-dominant inflammatory and the later Ly-6C^{low} macrophage-dominant reparative phases in the infarcted myocardium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Michael Waring and Adam Chicoine for sorting cells (Harvard Medical School). The authors thank Melissa Greene for technical assistance.

SOURCES OF FUNDING

This work was supported in part by NIH grants 1R01HL095612 and R56AI104695 (to F.K.S.). I.H. was supported by the DFG postdoctoral fellowship and the GTH Rudolf-Marx scholarship. L.M.S.G. was supported by the Boehringer Ingelheim Fonds and the German Cardiac Society.

Nonstandard Abbreviations and Acronyms

Arg1	arginase 1
BrdU	bromodeoxyuridine
Ccl	C–C chemokine ligand
Ccr	C–C chemokine receptor
CD	cluster of differentiation
ED	end diastolic
ES	end systolic
FS	fractional shortening
IL	interleukin
LAD	left anterior descending coronary artery
LPS	lipopolysaccharide
LVID	left ventricular inner diameter
Ly-6C	lymphocyte antigen 6C
Mac1	macrophage antigen 1
MI	myocardial infarction
Mo	monocyte
MΦ	macrophage
Nr4a1	nuclear receptor subfamily 4, group a, member 1
PSGL1	P-selectin glycoprotein ligand-1
TGF	tissue growth factor
Tlr	toll like receptor
TNF	tumor necrosis factor
TUNEL	TdT-mediated dUTP nick end labeling

VEGF vascular endothelial growth factor
WT wild type

References

- Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De Leon FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012; 380:2095–2128. [PubMed: 23245604]
- Bloom, DE.; Cafiero, ET.; Jane-Llopis, E.; Abrahams-Gessel, S.; Bloom, LR.; Fathima, S.; Feigl, AB.; Gaziano, T.; Mowafi, M.; Pandya, A.; Prettnner, K.; Rosenberg, L.; Seligman, B.; Stein, AZ.; Weinstein, C. The Global Economic Burden of Noncommunicable Diseases. Geneva: World Economic Forum; 2011.
- van Amerongen MJ, Harmsen MC, van Rooijen N, Petersen AH, van Luyn MJ. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am J Pathol*. 2007; 170:818–829. [PubMed: 17322368]
- Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation*. 2010; 121:2437–2445. [PubMed: 20530020]
- Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. *Circ Res*. 2012; 110:159–173. [PubMed: 22223212]
- Zouggari Y, Ait-Oufella H, Bonnin P, Simon T, Sage AP, Guerin C, Vilar J, Caligiuri G, Tsiantoulas D, Laurans L, Dumeau E, Kotti S, Bruneval P, Charo IF, Binder CJ, Danchin N, Tedgui A, Tedder TF, Silvestre JS, Mallat Z. B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. *Nat Med*. 2013; 19:1273–1280. [PubMed: 24037091]
- Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou-Khamis T, Michael LH, Rollins BJ, Entman ML, Frangogiannis NG. CCL2/Monocyte Chemoattractant Protein-1 regulates inflammatory responses critical to healing myocardial infarcts. *Circ Res*. 2005; 96:881–889. [PubMed: 15774854]

8. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009; 325:612–616. [PubMed: 19644120]
9. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med*. 2007; 204:3037–3047. [PubMed: 18025128]
10. Swirski FK, Nahrendorf M. Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. *Science*. 2013; 339:161–166. [PubMed: 23307733]
11. Wan E, Yeap XY, Dehn S, Terry R, Novak M, Zhang S, Iwata S, Han X, Homma S, Drosatos K, Lomasney J, Engman DM, Miller SD, Vaughan DE, Morrow JP, Kishore R, Thorp EB. Enhanced efferocytosis of apoptotic cardiomyocytes through myeloid-epithelial-reproductive tyrosine kinase links acute inflammation resolution to cardiac repair after infarction. *Circ Res*. 2013; 113:1004–1012. [PubMed: 23836795]
12. Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med*. 2007; 204:1057–1069. [PubMed: 17485518]
13. Avraham-Davidi I, Yona S, Grunewald M, Landsman L, Cochain C, Silvestre JS, Mizrahi H, Faroja M, Strauss-Ayali D, Mack M, Jung S, Keshet E. On-site education of VEGF-recruited monocytes improves their performance as angiogenic and arteriogenic accessory cells. *J Exp Med*. 2013; 210:2611–2625. [PubMed: 24166715]
14. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, Hartland SN, Snowden VK, Cappon A, Gordon-Walker TT, Williams MJ, Dunbar DR, Manning JR, van Rooijen N, Fallowfield JA, Forbes SJ, Iredale JP. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A*. 2012; 109:E3186–E3195. [PubMed: 23100531]
15. Varga T, Mounier R, Gogolak P, Poliska S, Chazaud B, Nagy L. Tissue LyC6- Macrophages Are Generated in the Absence of Circulating LyC6- Monocytes and Nur77 in a Model of Muscle Regeneration. *J Immunol*. 2013; 191:5695–5701. [PubMed: 24133167]
16. Li QX, Ke N, Sundaram R, Wong-Staal F. NR4A1, 2, 3--an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. *Histol Histopathol*. 2006; 21:533–540. [PubMed: 16493583]
17. McMorro JP, Murphy EP. Inflammation: a role for NR4A orphan nuclear receptors? *Biochem Soc Trans*. 2011; 39:688–693. [PubMed: 21428963]
18. Pei L, Castrillo A, Tontonoz P. Regulation of macrophage inflammatory gene expression by the orphan nuclear receptor Nur77. *Mol Endocrinol*. 2006; 20:786–794. [PubMed: 16339277]
19. Zhao Y, Bruemmer D. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. *Arterioscler Thromb Vasc Biol*. 2010; 30:1535–1541. [PubMed: 20631354]
20. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA, Geissmann F, Hedrick CC. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat Immunol*. 2011; 12:778–785. [PubMed: 21725321]
21. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007; 317:666–670. [PubMed: 17673663]
22. Carlin LM, Stamatides EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, Hedrick CC, Cook HT, Diebold S, Geissmann F. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell*. 2013; 153:362–375. [PubMed: 23582326]
23. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D’Cruz D, Casanova JL, Trouillet C, Geissmann F. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*. 2010; 33:375–386. [PubMed: 20832340]

24. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol.* 2004; 172:4410–4417. [PubMed: 15034056]
25. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guillemins M, Misharin A, Hume DA, Perlman H, Malissen B, Zelzer E, Jung S. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* 2013; 38:79–91. [PubMed: 23273845]
26. Pei L, Castrillo A, Chen M, Hoffmann A, Tontonoz P. Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. *J Biol Chem.* 2005; 280:29256–29262. [PubMed: 15964844]
27. Leuschner F, Rauch PJ, Ueno T, Gorbатов R, Marinelli B, Lee WW, Dutta P, Wei Y, Robbins C, Iwamoto Y, Sena B, Chudnovskiy A, Panizzi P, Keliher E, Higgins JM, Libby P, Moskowitz MA, Pittet MJ, Swirski FK, Weissleder R, Nahrendorf M. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *J Exp Med.* 2012; 209:123–137. [PubMed: 22213805]
28. Leuschner F, Dutta P, Gorbатов R, Novobrantseva TI, Donahoe JS, Courties G, Lee KM, Kim JJ, Markmann JF, Marinelli B, Panizzi P, Lee WW, Iwamoto Y, Milstein S, Epstein-Barash H, Cantley W, Wong J, Cortez-Retamozo V, Newton A, Love K, Libby P, Pittet MJ, Swirski FK, Kotliansky V, Langer R, Weissleder R, Anderson DG, Nahrendorf M. Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat Biotechnol.* 2011; 29:1005–1010. [PubMed: 21983520]
29. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol.* 2006; 7:311–317. [PubMed: 16462739]
30. Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest.* 2007; 117:902–909. [PubMed: 17364026]
31. Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo JL, Gorbатов R, Sukhova GK, Gerhardt LM, Smyth D, Zavitz CC, Shikatani EA, Parsons M, Rooijen NV, Lin HY, Husain M, Libby P, Nahrendorf M, Weissleder R, Swirski FK. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med.* 2013; 19:1166–1172. [PubMed: 23933982]
32. Zhu SN, Chen M, Jongstra-Bilen J, Cybulsky MI. GM-CSF regulates intimal cell proliferation in nascent atherosclerotic lesions. *J Exp Med.* 2009; 206:2141–2149. [PubMed: 19752185]
33. Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, MacDonald AS, Allen JE. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science.* 2011; 332:1284–1288. [PubMed: 21566158]
34. Hamers AA, Vos M, Rassam F, Marinkovic G, Kurakula K, van Gorp PJ, de Winther MP, Gijbels MJ, de Waard V, de Vries CJ. Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circ Res.* 2012; 110:428–438. [PubMed: 22194623]
35. Hanna RN, Shaked I, Hubbeling HG, Punt JA, Wu R, Herrley E, Zaugg C, Pei H, Geissmann F, Ley K, Hedrick CC. NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circ Res.* 2012; 110:416–427. [PubMed: 22194622]
36. Bonta PI, van Tiel CM, Vos M, Pols TW, van Thienen JV, Ferreira V, Arkenbout EK, Seppen J, Spek CA, van der Poll T, Pannekoek H, de Vries CJ. Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses. *Arterioscler Thromb Vasc Biol.* 2006; 26:2288–2294. [PubMed: 16873729]
37. Bonta PI, Matlung HL, Vos M, Peters SL, Pannekoek H, Bakker EN, de Vries CJ. Nuclear receptor Nur77 inhibits vascular outward remodelling and reduces macrophage accumulation and matrix metalloproteinase levels. *Cardiovasc Res.* 2010; 87:561–568. [PubMed: 20189954]
38. Chao LC, Soto E, Hong C, Ito A, Pei L, Chawla A, Conneely OM, Tangirala RK, Evans RM, Tontonoz P. Bone marrow NR4A expression is not a dominant factor in the development of atherosclerosis or macrophage polarization in mice. *J Lipid Res.* 2013; 54:806–815. [PubMed: 23288947]
39. Cheng Z, Volkens M, Din S, Avitabile D, Khan M, Gude N, Mohsin S, Bo T, Truffa S, Alvarez R, Mason M, Fischer KM, Konstandin MH, Zhang XK, Heller Brown J, Sussman MA. Mitochondrial

- translocation of Nur77 mediates cardiomyocyte apoptosis. *Eur Heart J.* 2011; 32:2179–2188. [PubMed: 21228009]
40. You B, Jiang YY, Chen S, Yan G, Sun J. The orphan nuclear receptor Nur77 suppresses endothelial cell activation through induction of IkappaBalpha expression. *Circ Res.* 2009; 104:742–749. [PubMed: 19213954]
 41. Cunningham NR, Artim SC, Fornadel CM, Sellars MC, Edmonson SG, Scott G, Albino F, Mathur A, Punt JA. Immature CD4+CD8+ thymocytes and mature T cells regulate Nur77 distinctly in response to TCR stimulation. *J Immunol.* 2006; 177:6660–6666. [PubMed: 17082578]
 42. Fassett MS, Jiang W, D'Alise AM, Mathis D, Benoist C. Nuclear receptor Nr4a1 modulates both regulatory T-cell (Treg) differentiation and clonal deletion. *Proc Natl Acad Sci U S A.* 2012; 109:3891–3896. [PubMed: 22345564]
 43. Swirski FK, Robbins CS. Neutrophils usher monocytes into sites of inflammation. *Circ Res.* 2013; 112:744–745. [PubMed: 23449542]
 44. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat Immunol.* 2013; 14:986–995. [PubMed: 24048120]
 45. Sieweke MH, Allen JE. Beyond stem cells: self-renewal of differentiated macrophages. *Science.* 2013; 342:1242974. [PubMed: 24264994]
 46. Frangogiannis NG, Mendoza LH, Ren G, Akrivakis S, Jackson PL, Michael LH, Smith CW, Entman ML. MCSF expression is induced in healing myocardial infarcts and may regulate monocyte and endothelial cell phenotype. *Am J Physiol Heart Circ Physiol.* 2003; 285:H483–H492. [PubMed: 12689859]
 47. Anversa P, Kajstura J, Rota M, Leri A. Regenerating new heart with stem cells. *J Clin Invest.* 2013; 123:62–70. [PubMed: 23281411]
 48. Chen W, Frangogiannis NG. Fibroblasts in post-infarction inflammation and cardiac repair. *Biochim Biophys Acta.* 2013; 1833:945–953. [PubMed: 22982064]
 49. Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nat Rev Mol Cell Biol.* 2013; 14:529–541. [PubMed: 23839576]
 50. Zamilpa R, Lindsey ML. Extracellular matrix turnover and signaling during cardiac remodeling following MI: causes and consequences. *J Mol Cell Cardiol.* 2010; 48:558–563. [PubMed: 19559709]

Novelty and Significance

What Is Known?

- Transcription factor Nr4a1 is essential to Ly-6C^{low} but not Ly-6C^{high} monocyte development.
- Accumulation of monocytes and macrophages in the infarcted myocardium is biphasic, involving an early Ly-6C^{high} inflammatory phase and a later Ly-6C^{low} reparative phase.
- The inflammatory response is exaggerated in LPS-stimulated Nr4a1-deficient bone marrow-derived macrophages.

What New Information Does This Article Contribute?

- Ly-6C^{high} monocytes give rise to both the Ly-6C^{high} inflammatory and Ly-6C^{low} reparative phases.
- Nr4a1-expression limits CCR2-mediated recruitment of Ly-6C^{high} monocytes to the infarcting tissue.
- Ly-6C^{low} macrophages derive from Ly-6C^{high} monocytes, proliferate, and utilize Nr4a1 to dampen inflammation.

Myocardial infarction involves accumulation of monocytes and macrophages in tissue. Monocytes in mice comprise at least two functionally distinct subsets exhibiting different expression of Ly-6C. The orphan nuclear receptor Nr4a1 is essential to the development of Ly-6C^{low} but not Ly-6C^{high} monocytes. We therefore utilized Nr4a1-deficient mice to study how Ly-6C^{low} monocytes and Nr4a1 expression in cardiac macrophages contribute to myocardial infarct healing. Ly-6C^{low} monocytes accumulated at low numbers in the infarct tissue and did not differentiate into cardiac macrophages. In contrast, inflammatory Ly-6C^{high} monocytes dominated the infiltrate within the first three days after induction of myocardial infarction, differentiated into Ly-6C^{low} macrophages, dominated the second healing phase, and proliferated. This process coincided with elevated Nr4a1 expression in cardiac macrophages and increased expression of regenerative factors. Nr4a1 expression, albeit lower in Ly-6C^{high} monocytes, corresponded with suppressed expression of chemokine receptor CCR2, 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.

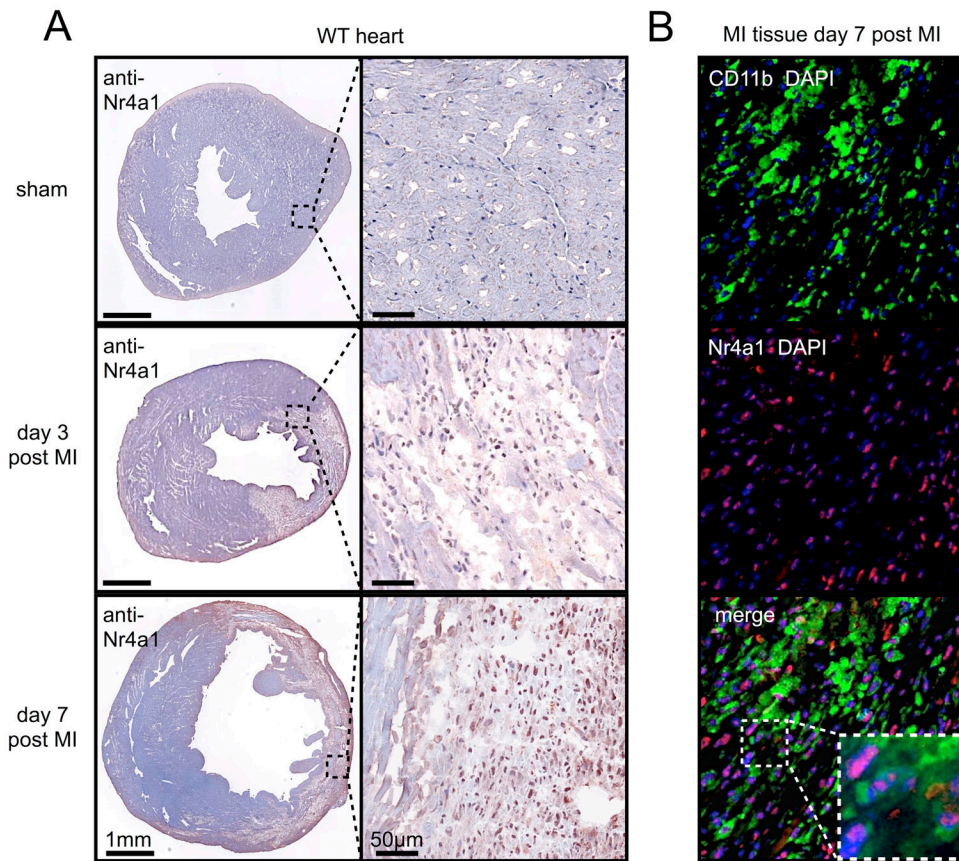


Figure 1. Nr4a1 expressing myeloid cells accumulate in myocardial infarction tissue
A. Cross sections of heart tissue 3 and 7 days after permanent ligation of the left anterior descending coronary artery (LAD) or sham surgery. Overview and high magnification images of immunohistochemical staining for Nr4a1. **B.** Immunofluorescence co-staining for Nr4a1 and CD11b of MI tissue 7 days after coronary artery ligation. Representative images from one out of three samples are shown.

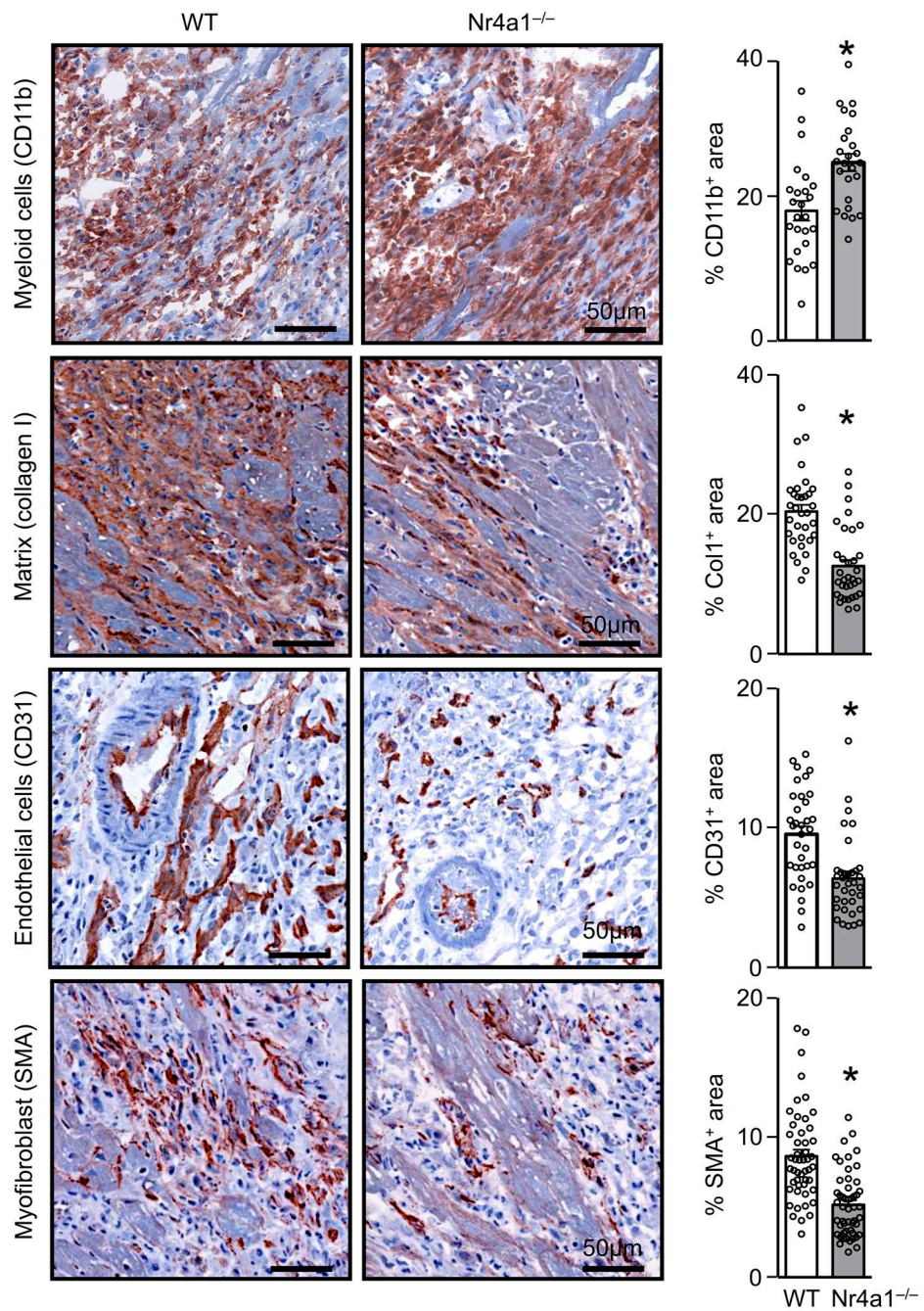


Figure 2. Adverse cardiac remodeling in *Nr4a1*^{-/-} chimeric mice 7 days after myocardial infarction

Immunohistochemical staining of MI tissue for CD11b, Collagen 1 (Col1), CD31, and non-vascular smooth muscle alpha actin (SMA) in wild-type (WT) and *Nr4a1*^{-/-} chimeric mice 7 days post MI. Quantification of 10 randomly selected fields of view per sample. Results are presented as mean ± SEM, * p < 0.05, n = 5 per group.

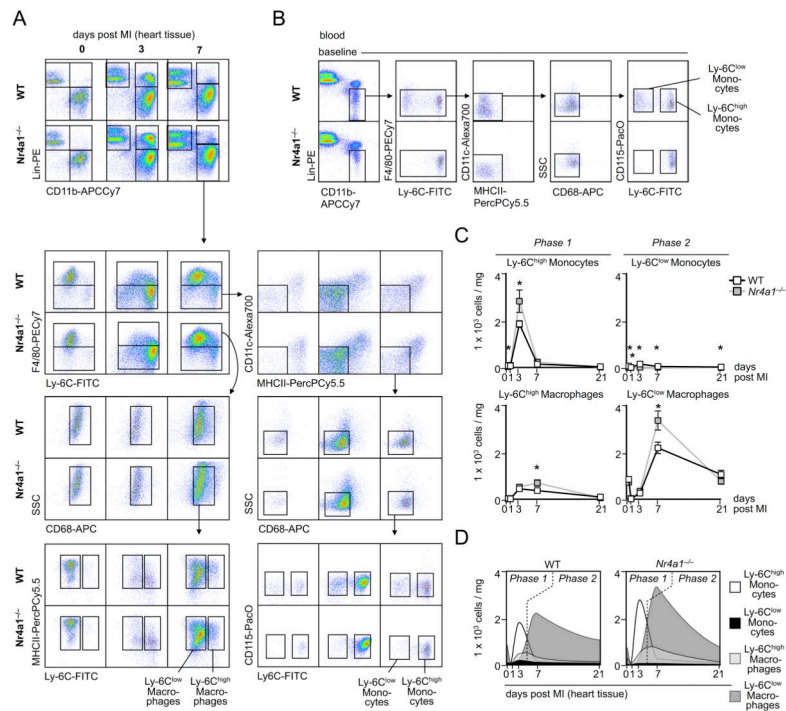


Figure 3. Enhanced accumulation of *Nr4a1*^{-/-} monocytes and macrophages in myocardial infarct tissue

A. Representative images for flow cytometric analysis of MI tissue cell suspensions before, 3 and 7 days after permanent LAD ligation in 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 versus *Nr4a1*^{-/-} mice before and 1, 3, 7, and 21 days post 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.

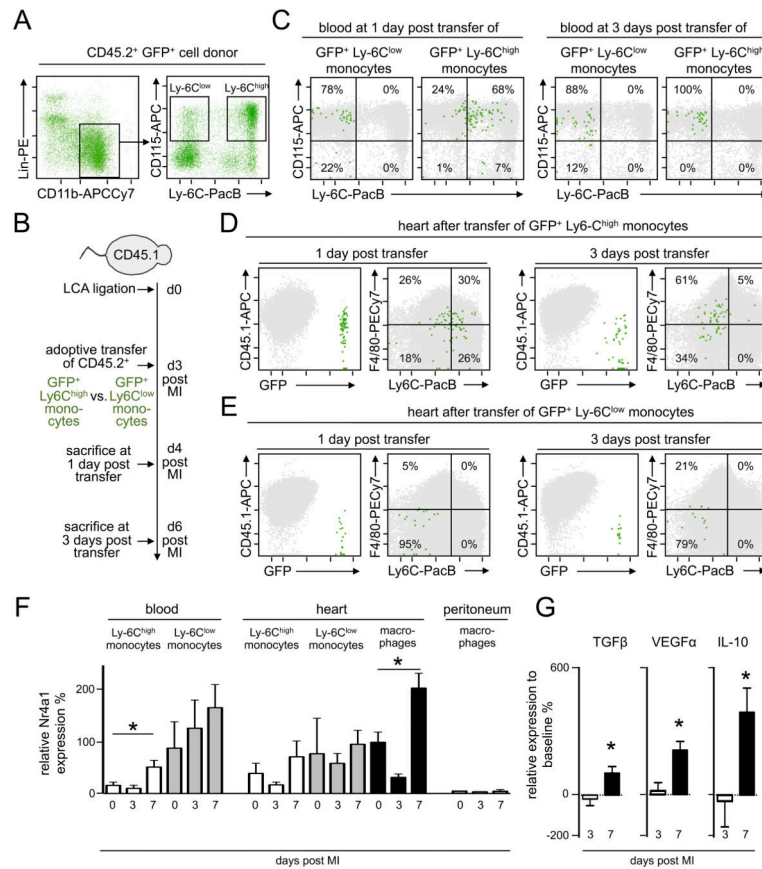


Figure 4. Nr4a1^{low} Ly-6C^{high} monocytes give rise to Nr4a1^{high} Ly-6C^{low} macrophages in myocardial infarct tissue

A. Identification of Ly-6C^{high} and Ly-6C^{low} monocyte subsets in pooled CD45.2⁺ GFP⁺ blood and spleen samples for flow assisted cell sorting. **B.** Illustration of experimental approach with separate adoptive transfer of sorted GFP⁺ Ly-6C^{high} monocytes (1×10^6) and GFP⁺ Ly-6C^{low} monocytes (0.5×10^6) 3 days after permanent ligation of the LAD in CD45.1⁺ mice. Sacrifice 1 and 3 days after adoptive transfer. **C.** Flow cytometric analysis of blood from CD45.1⁺ recipients of GFP⁺ monocyte subsets on days 1 and 3 after transfer. Quantification of the percentage of GFP⁺ cells within the respective gates. Pooled data of two independent experiments. **D.** Flow cytometric analysis of MI tissue from CD45.1⁺ recipients of GFP⁺ Ly-6C^{high} monocytes on days 1 and 3 after transfer. Quantification of the percentage of GFP⁺ cells within the respective gates. Pooled data of two independent experiments. **E.** Flow cytometric analysis of MI tissue from CD45.1⁺ recipients of GFP⁺ Ly-6C^{low} monocytes on days 1 and 3 after transfer. Quantification of the percentage of GFP⁺ cells within the respective gates. Pooled data of two independent experiments. **F.** Quantification (qRT-PCR) of Nr4a1 expression in cells sorted from the blood, MI tissue and unstimulated peritoneal lavage on days 3 and 7 after permanent LAD ligation compared to sham operated C57Bl/6 mice (0 days post MI). Results are presented as mean \pm SEM, * p 0.05, n = 4 per time point. **G.** Relative expression of VEGF α , TGF β and IL-10 in sorted Ly-6C^{high} monocytes on day 3 (phase 1) and macrophages on day 7 (phase 2) post MI

compared to baseline cardiac macrophages. Results are presented as mean \pm SEM, * p 0.05, n = 4 per time point.

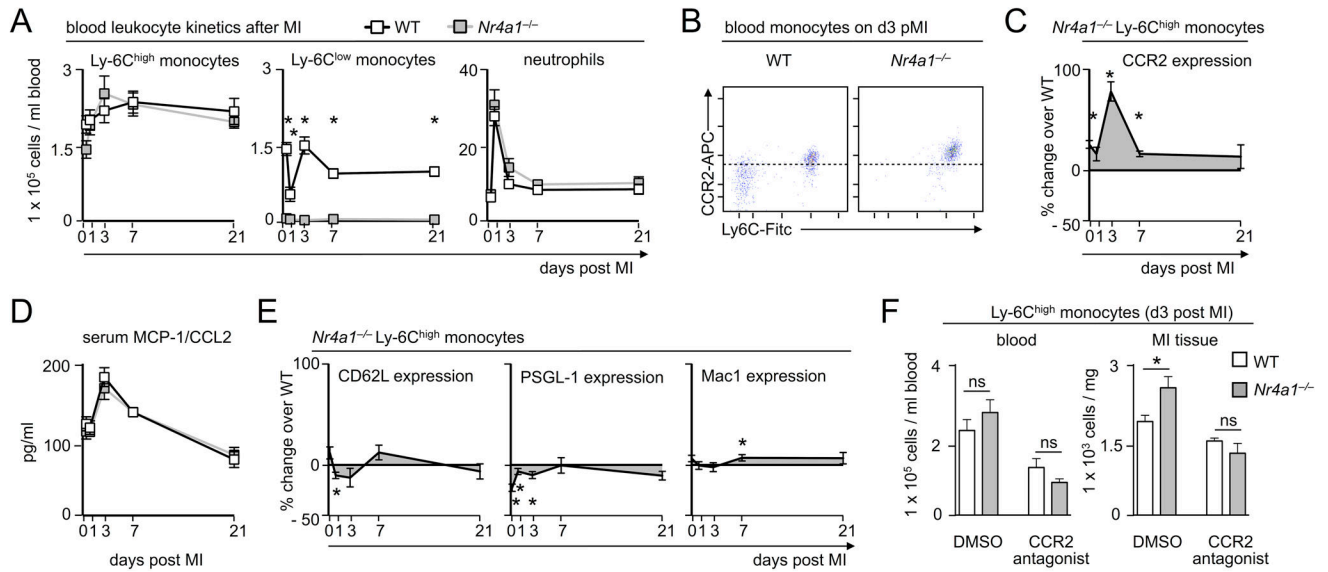


Figure 5. *Nr4a1*-deficiency promotes CCR2-mediated monocyte recruitment to the myocardial infarct tissue

A. Quantification of monocyte subsets and neutrophils in the blood of WT and *Nr4a1*^{-/-} mice before and on days 1, 3, 7 and 21 post MI. Results are presented as mean \pm SEM, * $p < 0.05$, $n = 5$ 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 post MI. **C.** Quantification of CCR2 expression on Ly-6C^{high} monocytes by mean fluorescence intensity at indicated time points after MI. Results are shown as mean \pm SEM percent change of CCR2 expression in *Nr4a1*^{-/-} compared to WT control mice, * $p < 0.05$, $n = 5$ per group and time point. **D.** Quantification of serum MCP-1/CCL2 levels in WT and *Nr4a1*^{-/-} mice before and on days 1, 3, 7 and 21 post MI. Results are presented as mean \pm SEM, $n = 5$ 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-6C^{high} monocytes by mean fluorescence intensity at indicated time points after MI. Results are shown as mean \pm SEM percent change of marker expression in *Nr4a1*^{-/-} compared to WT control mice, * $p < 0.05$, $n = 5$ per group and time point. **F.** Quantification of Ly-6C^{high} monocyte numbers in peripheral blood and MI tissue on day 3 after permanent LAD ligation in WT and *Nr4a1*^{-/-} mice treated with CCR2 antagonist (RS504393, 2mg/kg ip. b.i.d.) or vehicle (30% DMSO ip) alone. Results are presented as mean \pm SEM, * $p < 0.05$, $n = 5$ per group.

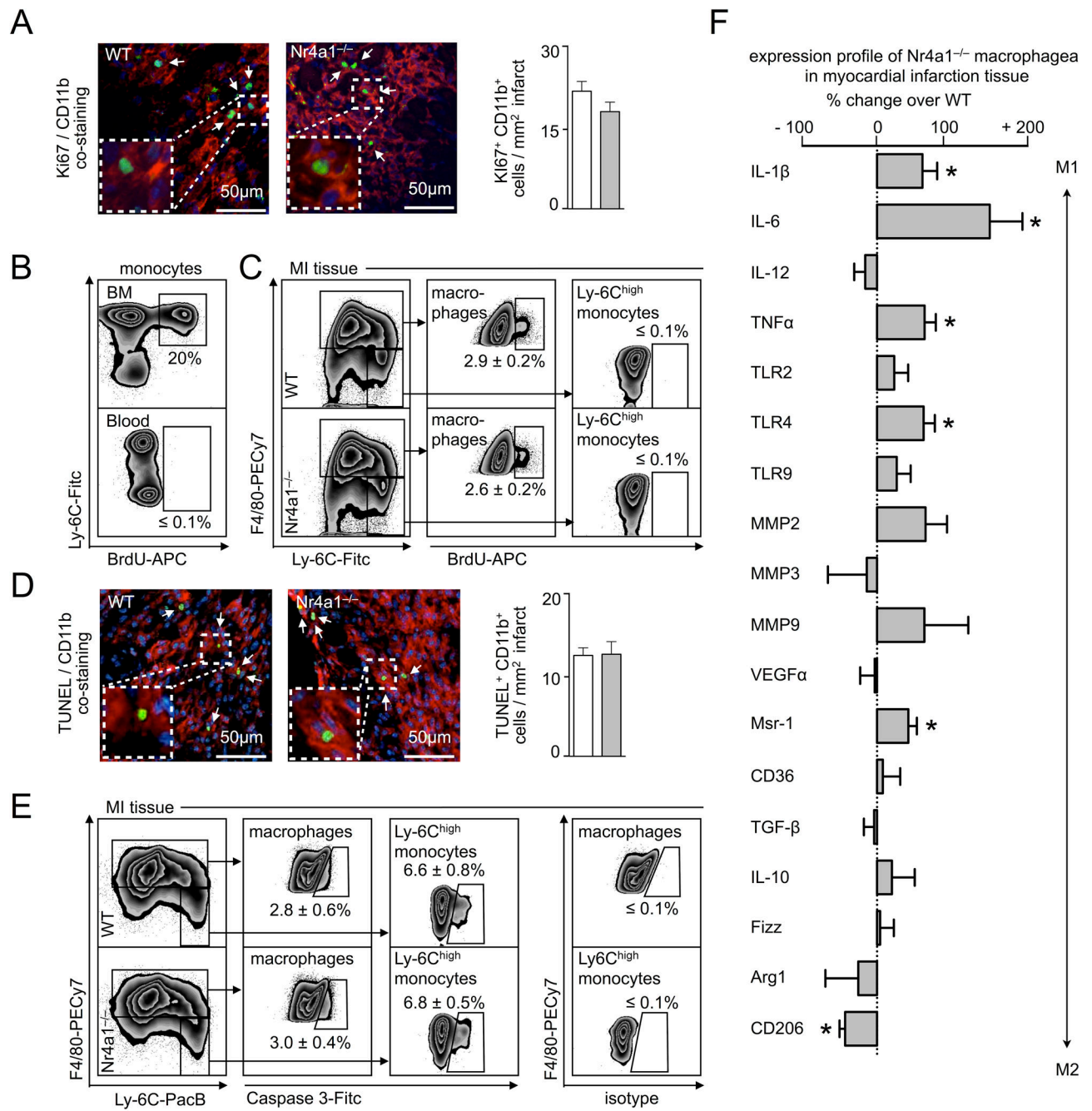


Figure 6. Nr4a1-deficient macrophages are pro-inflammatory, proliferate and die in infarct tissue

A. Quantification of Ki67⁺ CD11b⁺ cells in MI tissue 7 days after permanent LAD ligation in WT and Nr4a1^{-/-} mice on the right and representative merged images for immunofluorescent co-staining on the left. Results are presented as mean ± SEM, n=5 per group. **B.** Flow cytometric analysis of BrdU incorporation into WT blood and bone marrow (BM) monocytes 2 hours after iv injection of 200µg BrdU. **C.** Flow cytometric analysis of BrdU incorporation into WT and Nr4a1^{-/-} Ly-6C^{high} monocytes and macrophages in MI

tissue 7 days after permanent LAD ligation. Results are presented as mean \pm SEM, n =5 per group. **D.** Quantification of TUNEL⁺ CD11b⁺ cells in MI tissue 7 days after permanent LAD ligation in WT and *Nr4a1*^{-/-} mice on the right and representative merged images for immunofluorescent co-staining on the left. Results are presented as mean \pm SEM, n =5 per group. **E.** Flow cytometric analysis of Caspase 3 expression in WT and *Nr4a1*^{-/-} Ly-6C^{high} monocytes and macrophages in MI tissue 7 days after permanent LAD ligation. Results are presented as mean \pm SEM, n =5 per group. Isotype staining is shown on the right. **F.** Gene expression profiling of WT and *Nr4a1*^{-/-} macrophages sorted from MI tissue 7 days after permanent LAD ligation. Results are presented as mean \pm SEM percent change of marker expression in *Nr4a1*^{-/-} compared to WT control mice, * p < 0.05, n = 5 per group.

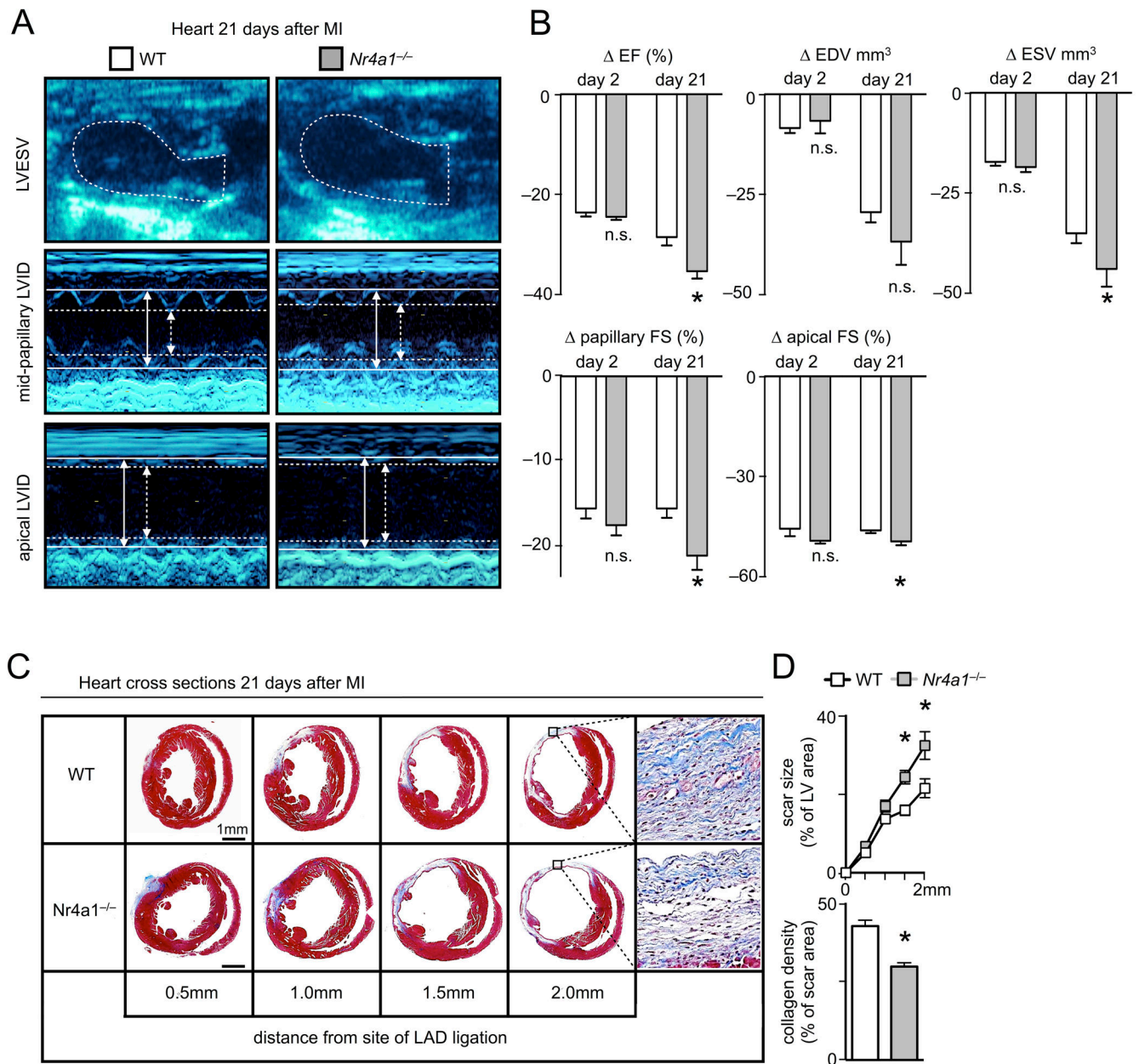


Figure 7. Nr4a1 limits left ventricular dysfunction after myocardial infarction

A. Representative echocardiography images with long axis B-mode (upper panel) and short axis M-mode views (lower panels) of infarcted hearts from WT and *Nr4a1*^{-/-} mice on day 21 after permanent LAD ligation. Arrows and lines mark left ventricular inner diameters (LVID) in systole (dashed) and diastole (firm). **B.** Quantification of individual changes () in heart parameters compared to baseline on days 2 and 21 post MI. Results are presented as mean \pm SEM percent change of marker expression in *Nr4a1*^{-/-} compared to WT control mice, * $p < 0.05$, $n = 8$ per group. **C.** Representative images of heart cross sections 21 days post MI at increasing distance from the site of coronary artery ligation in *Nr4a1*^{-/-} and WT control mice, $n = 5$ per group. **D.** Quantification of scar size in heart cross sections at

different levels depending on the distance from the site of coronary artery ligation. Data are presented as a fraction of the left ventricle including the septum, mean \pm SEM, * $p < 0.05$, $n = 5$ per group. Collagen density is quantified in 5 randomly selected fields of view per sample within the scar at 2 mm distance from the site of coronary artery ligation. Results are presented as mean \pm SEM, * $p < 0.05$, $n = 5$ per group.

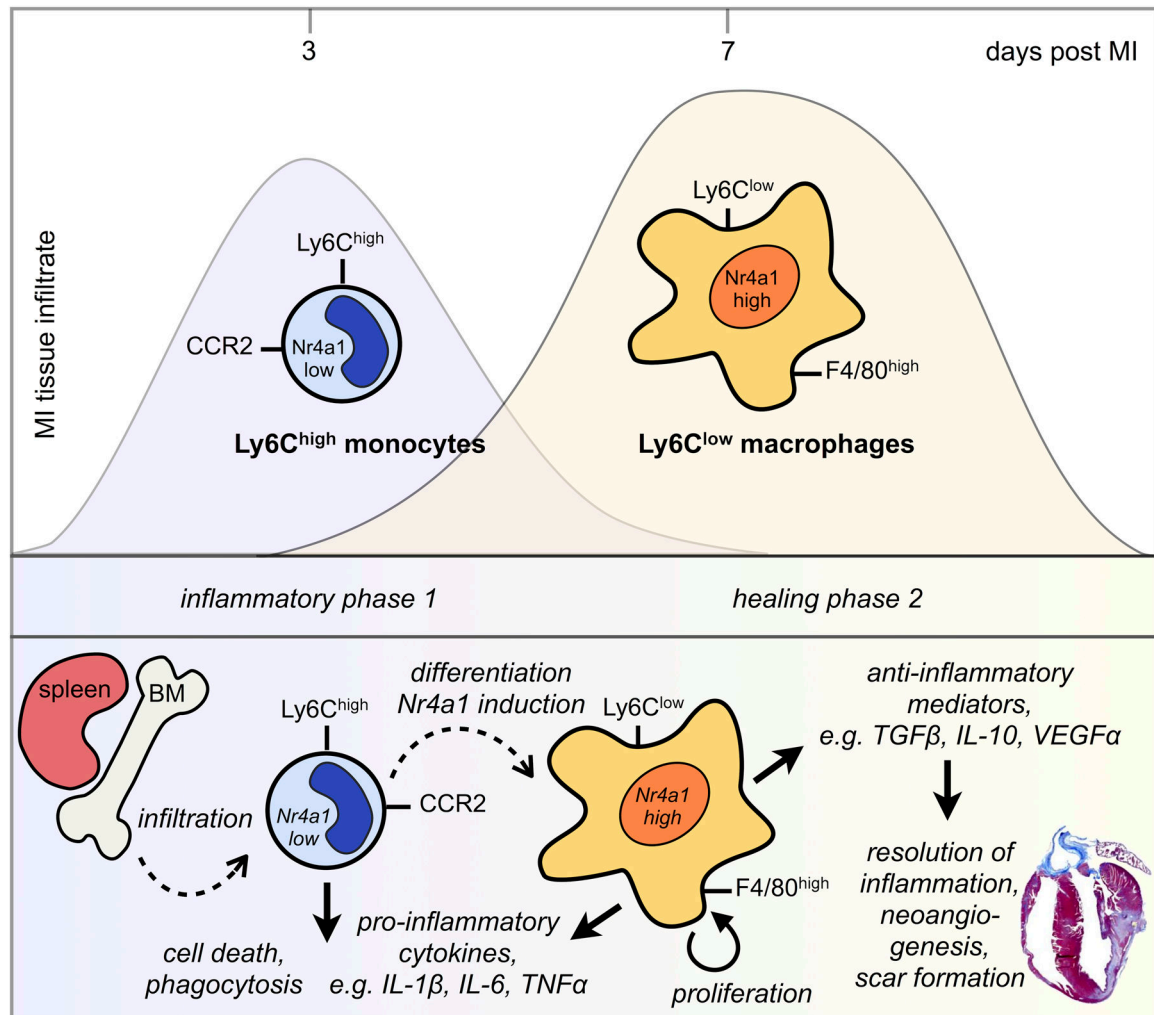


Figure 8. Biphasic model of monocyte/macrophage accumulation in MI tissue

During the early phase of myocardial infarct healing inflammatory $\text{Nr4a1}^{\text{low}}$ $\text{Ly-6C}^{\text{high}}$ monocytes from the bone marrow and spleen infiltrate the MI tissue via CCR2. Consequently they differentiate into proliferating $\text{Nr4a1}^{\text{high}}$ $\text{Ly-6C}^{\text{low}}$ macrophages that participate in the resolution of inflammation and cardiac remodeling in the second phase of infarct healing.