Differential Contribution of Monocytes to Heart Macrophages in Steady-State and after Myocardial Infarction

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Differential Contribution of Monocytes to Heart Macrophages in Steady-State and after Myocardial Infarction

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Abstract

Rationale—Macrophages populate the steady-state myocardium. Previously, all macrophages were thought to arise from monocytes; however, it emerged that in several organs tissue-resident macrophages may self-maintain through local proliferation.

Objective—To study the contribution of monocytes to cardiac resident macrophages in steady-state, after macrophage depletion in CD11b$^{DTR/+}$ mice and in myocardial infarction.

Methods and Results—Using in vivo fate mapping and flow cytometry, we estimated that during steady-state the heart macrophage population turns over in about one month. To explore the source of cardiac resident macrophages, we joined the circulation of mice using parabiosis. After 6 weeks, we observed blood monocyte chimerism of 35.3±3.4% while heart macrophages showed a much lower chimerism of 2.7±0.5% ($p<0.01$). Macrophages self renewed locally through proliferation: 2.1±0.3% incorporated BrdU 2 hours after a single injection and 13.7±1.4% heart macrophages stained positive for the cell cycle marker Ki67. The cells likely participate in defense against infection, as we found them to ingest fluorescently labeled bacteria. In ischemic myocardium, we observed that tissue resident macrophages died locally while some also migrated to hematopoietic organs. If the steady-state was perturbed by coronary ligation or diphtheria toxin-induced macrophage depletion in CD11b$^{DTR/+}$ mice, blood monocytes replenished heart macrophages. However, in the chronic phase after myocardial infarction, macrophages residing in the infarct were again independent from the blood monocyte pool, returning to the steady-state situation.

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DISCLOSURES
None.

Conclusions—In this study we show differential contribution of monocytes to heart macrophages during steady-state, after macrophage depletion or in the acute and chronic phase after myocardial infarction. We found that macrophages participate in the immunosurveillance of myocardial tissue. These data correspond with previous studies on tissue-resident macrophages and raise important questions on the fate and function of macrophages during the development of heart failure.

Keywords

Macrophage; monocyte; heart; myocardial infarction; myocardial

INTRODUCTION

Macrophages are innate immune cells that pursue a plethora of functions in steady-state and disease. The name “big eater” refers to their uptake of invading pathogens, dying cells, foreign bodies and other materials, including cholesterol. The macrophage phenotype and their tissue-specific functions depend on the environment they reside in. For instance, macrophages may contribute to the regulation of thermogenesis, influence the electrolyte balance or iron recycling[1]. Fate mapping studies revealed a dichotomy for macrophage’s sources: contrary to previous dogma, tissue-resident macrophages in the brain, liver, lung and skin do not derive from monocytes that circulate in the blood but are replenished through local proliferation[2–5]. Tissue-resident macrophages in the intestine and inflammatory macrophages in sites of acute inflammation, however, derive from monocytes that were produced in the bone marrow or in the spleen[6, 7].

Recently, it emerged that macrophages populate the healthy and diseased myocardium. While their higher numbers and density rendered them an easy to discern and well-studied cell population in acutely ischemic heart tissue[8], their interspersed position in between myocytes, fibroblasts and endothelial cells in the steady-state myocardium was a likely reason why the cells previously escaped detection. The advent of more sensitive imaging tools and genetic reporter proteins, together with methods to detect even sparse leukocyte populations in tissue by flow cytometry, lead to an increased appreciation of macrophages’ presence in the healthy and also in chronically diseased myocardium[9–12]. Given the recent description of macrophages in the murine myocardium, we are just beginning to understand the cells’ functions in the steady-state heart and in heart failure. For other organs, there is a rich body of knowledge on macrophages’ role in health and disease, indicating that these cells promote tissue destruction, fibrosis, angiogenesis and instruct local tissue progenitors.

Here we investigated the maintenance of cardiac resident macrophages by assessing cell turnover as well as the contribution of bone marrow-derived monocytes to the pool of heart macrophages in steady-state and disease. Steady-state heart macrophages proliferate locally and self renew independently of circulating monocytes. However, monocytes contribute to macrophages in the acute infarct or after induction of macrophage death in CD11b$^{DTR/+}$ mice.

METHODS

Animals and procedures

All experimental animal procedures performed were approved by the Subcommittee on Animal Research Care at Massachusetts General Hospital (13th Street, Charlestown, MA, USA). All experiments were performed with 10–12 weeks old female animals. C57BL/6J (stock 000664), hemizygous B6.129P-Cx3cr1^{tm1Litt}/J (cx3cr1$^{GFP/+}$, stock 005582), B6.FVB-Tg (ITGAM-DTR/EGFP) 34Lan/J (stock 006000), C57BL/6-Tg(UBC-GFP) 30Scha/J (stock 004353) and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ mice (stock 002014) used in this study were purchased from Jackson laboratory. C57BL/6-αSMA^{RFP} were a kind gift from David Brenner. Mice were joined by parabiosis using either CD45.2^{+} and GFP^{+} mice or CD45.1^{+} and CD45.2^{+} mice[13]. Fate mapping studies used wild type and not Cx3cr1$^{GFP/+}$ mice to exclude disturbance of cell recruitment due to receptor haploinsufficiency. Macrophage depletion was achieved by a single intraperitoneal injection of diphteria toxin (DT) in CD11b$^{DTR/+}$ mice (25 ng/g body weight, Sigma). We used C57BL/6 mice injected with DT or non injected CD11b$^{DTR/+}$ mice as controls. Myocardial infarction was induced by permanent coronary ligation as previously described[14]. Briefly, mice were anesthetized, given buprenorphin s.c. for analgesia, intubated and ventilated with 2% isoflurane supplemented with oxygen. After thoracotomy, the heart was exposed, the left coronary artery was identified and permanently ligated with a monofilament nylon 8-0 suture.

Cell preparation and flow cytometry

Single cell suspensions were obtained from peripheral blood, heart, spleen, bone marrow and lung. Briefly, blood was collected by cardiac puncture in 50mM EDTA and red blood cells were lysed with 1× red blood cell lysis buffer (Biolegend). Mice were perfused trough the left ventricle with 30ml of ice-cold PBS. Bone marrow was harvested by flushing femurs in 0.5% bovine serum albumin and 1% fetal bovine serum (FACS buffer). Lung and Heart were excised, minced with a fine scissor prior to digestion in 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I and 60 U/ml hyaluronidase (Sigma-Aldrich) for 1 hour at 37°C under agitation. Tissues were triturated and cells filtered through a 40μm nylon mesh (BD Falcon), washed and centrifuged (8 min, 300 g, 4°C). Cells were stained at 4°C in PBS with FACS buffer. Fluorochrome- and biotin-conjugated antibodies specific to mouse CD90 (clone 53-2.1), B220 (clone RA3-6B2), CD19 (clone 1D3), CD49b (clone DX5), NK1.1 (clone PK136), Ly-6G (clone 1A8 and clone RB6-8C5), Ter-119 (clone TER-119), CD45.1 (clone A20), CD45.2 (clone 104), CD11c (clone HL3), CD11b (clone M1/70), CD115 (clone AFS98), F4/80 (clone BM8), Ly6C (clone AL-21 and HK1.4), IL7Rα (clone A7R34), c-kit (clone 2B8), Sca-1 (clone D7), CD48 (HM48-1), CD150 (clone TC15-12F12.2) and streptavidin Pacific Orange™ and Pacific Blue™ conjugates (Life technologies) were used. Antibodies were purchased from either eBioscience, Biolegend, or BD biosciences. Monocytes were identified as Lineage (CD90/CD19/NK1.1/Ly-6G/Ter119)^{low}, CD11b^{+}, F4/80 ^{low}, CD115^{high}, Ly-6C $^{low/high}$. Macrophages were identified as Lin (CD90/CD19/NK1.1/Ly-6G/Ter119) ^{int}, CD11b^{+}, F4/80^{high}, Ly-6C ^{low}. For BrdU incorporation assays, mice were injected intraperitoneally with 1 mg BrdU i.p. (Sigma) in 100μl PBS 2 hours prior to organ harvest. In another set of experiments to investigate macrophage turnover, mice were injected daily for 4 weeks. Intracellular staining was

performed using BrdU Flow Kits according to manufacturer's instructions (BD Biosciences). Mice that were not injected with BrdU served as controls. For cell cycle analysis with Ki67 intracellular staining, cells were fixed and permeabilized using the transcription factor staining buffer set (eBioscience) and stained with FITC anti-Ki67 antibody (ebioscience, clone SolA15). 1 μl DAPI (FxCycle™ Violet Stain, Life Technologies) was added directly prior to FACS analysis. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Bone marrow transplantation

For adoptive transfer experiments, recipient GFP^+ mice were lethally irradiated (10 Gy) and transplanted intravenously with one hundred FACS-sorted hematopoietic stem cells (SLAM HSC) defined as Lineage (CD90/B220/CD49b/NK1.1/Ly-6G/Ter119/CD11b/CD11c/IL7Rα)^{low} c-kit^{high} Sca-1^{high} CD48^{neg} CD150^{high} obtained from $CD45.2^+$ mice together with 2×10^5 supportive bone marrow cells from GFP^+ mice. Percent donor chimerism was evaluated in blood monocytes and heart macrophages 18 weeks later. For intravenous injection, cells were resuspended in 150 μl 0.9% saline buffer.

In vivo phagocytosis assay

C57BL/6 mice received four injections (10μg each) of pHrodo™ Green *Staphylococcus aureus* (2mg/ml, Life Technologies) into the left ventricular myocardium two hours prior to sacrifice. Green fluorescence of injected bacteria is only induced upon phagocytosis and contact to low pH in phagosomes. Uptake in target cells was assessed using flow cytometry of excised left ventricular myocardium.

Cell tracking

Vybrant® DiO cell-labeling solution (Molecular Probes®) was diluted 1:100 in PBS and 10μl were injected at five different spots into the myocardium prior to myocardial infarction induced by coronary ligation. Injected mice without subsequent MI or mice injected with PBS served as controls. Hearts, spleen, bone marrow and mediastinal lymph nodes were harvested 24 hours later and DiO labeled-macrophages were analyzed by flow cytometry.

Immunofluorescence microscopy

Hearts were removed, flushed with 0.9% saline buffer, fixed in 4% PFA (paraformaldehyde, methanol-free, Electron Microscopy Science) for 3 hours and immersed in 30 % sucrose in PBS overnight. Then tissue was embedded in Tissue-Tek® OCT compound (Sakura Finetek), and frozen in an isopentane bath on dry ice. For immunofluorescence staining, sections (25 μm) were stained with anti-MAC3 antibody (clone M3/84, BD Biosciences) followed by a biotinylated anti-rat secondary antibody and streptavidin-DyLight 594 (Vector Laboratories, Inc.). Fluorescence TUNEL staining was performed using DeadEnd Fluorometric TUNEL system (Promega) and fibroblasts in heart were detected using DDR2 antibody (LifeSpan BioSciences, Inc.). The sections were counterstained with DAPI (Life Technologies) to identify nuclei. Images were captured using a Nikon Eclipse 80i (Nikon Instruments) or slides were scanned with NanoZoomer 2.0-RS in high resolution mode (Hamamatsu, Japan).

Human myocardial histology

Post-mortem tissue specimens of the left ventricle were collected from 5 patients that were referred to the department of Pathology, VU University Medical Center (VUmc), Amsterdam, the Netherlands, for clinical autopsy (n = 5, male = 3, age = 58 ± 20 years). All 5 patients died from a cause not related to cardiac disease (acute aortic rupture/dissection, n = 3; chronic obstructive pulmonary disease, n = 1; trauma, n = 1. Tissue specimens were formalin-fixed and paraffin-embedded. Sections (4 μm thick) were stained with an antibody for human CD68 (clone KP1, 1:400, Dako, Glostrup, Denmark) to identify macrophages. The present study was conducted in accordance with the Declaration of Helsinki. The study protocol (CASIMIR) was approved by the Research Committee of the Department of Pathology of the VUmc. The use of autopsy material after completion of the diagnostic process is part of the patient contract in the VUmc.

Statistics

Data are expressed as mean±SEM. Analyses were performed using Prism 6.0a (GraphPad Software Inc.). For a two-group comparison, a student t-test was applied if the pre-test for normality (D'Agostino-Pearson normality test) was not rejected at 0.05 significance level, otherwise a Mann-Whitney test for nonparametric data was used. ANOVA tests, followed by Bonferroni post-tests was applied for comparison of more then 2 groups. P values of <0.05 indicate statistical significance.

RESULTS

Resident heart macrophage turnover is slow during steady-state

Using flow cytometry we estimated that leukocytes contribute 10.3±0.4% of all murine cardiac cells, and that $CD45^+$ $CD11b^+$ $F4/80^+$ $Ly6C^{low}$ macrophages are the most prominent population among cardiac leukocytes (7.9±0.3% of all cardiac cells, Fig. 1A). Previously, the Rosenthal group visualized cardiac resident macrophages in the healthy myocardium using fractalkine $Cx3cr1^{GFP/+}$ reporter mice[9]. As the fractalkine receptor may be expressed by various cell types, we investigated $Cx3cr1^{GFP}$ reporter expression by $CD45^-$ cells ($CD45^-$ cells include fibroblasts, endothelial cells and myocytes), lymphocytes and macrophages. We confirmed that $Cx3cr1^{GFP}$ is expressed by macrophages in the healthy murine heart while other cardiac cells, including $CD45^-$ fibroblasts, do not show Cx3cr1 expression by FACS (Fig. 1B). In addition, GFP^+ cells in $Cx3cr1^{GFP/+}$ mice expressed MAC3 (Fig. 1C) and F/80 (Fig. 1D) on multicolor fluorescence histology. Because heart macrophages' spindle-like shape resembles fibroblasts, we crossed $Cx3cr1^{GFP/+}$ mice with a $\alpha SMA^{RFP/+}$ mouse strain in which myofibroblasts are red. These dual reporter mice showed green and red protein reporter expression in different cells on fluorescence microscopy (Figure 1E), clearly separating $Cx3cr1^{GFP/+}$ macrophages from myofibroblasts. We further stained myocardium from $Cx3cr1^{GFP/+}$ mice for the fibroblasts marker DDR2, and likewise did not find colocalisation of GFP macrophage signal with fibroblasts (Fig. 1F). In a series of 5 autopsy cases, we stained human left ventricular myocardial tissue for the macrophage marker CD68. Macrophages were found in all examined human hearts. Depending on the orientation of myocardial fibers in the section, we observed similar spindle-like macrophage shapes in human myocardium (Fig. 1G). Finally, we FACS-sorted GFP^+ cells from two

Cx3cr1$^{GFP/+}$ hearts for a cytospin preparation. When placed on a microscopy slide, GFP$^+$ cells assumed the typical irregular round shape of macrophages (Fig. 1H).

To explore the turnover kinetics of cardiac resident macrophages, we performed a pulse-chase experiment with 5-Bromo-2′-Deoxyuridine (BrdU). After loading heart macrophages with BrdU by daily injections for four weeks (wash-in phase; pulse), saturation was measured in a first cohort. A similarly treated littermate cohort was assessed after an additional three week wash-out (chase) without further BrdU exposure. We found that the BrdU label in heart macrophages decreased from 40.1 ±1.4% at the end of the pulse phase to 15.5±2.0% at the end of the chase phase, reflecting cell turnover (Fig. 2). This 2.6 fold decrease of BrdU$^+$ macrophages within 3 weeks indicates that cardiac resident macrophages turn over slowly in steady-state. Extending on the 2.6-fold decrease BrdU$^+$ macrophages in three weeks, one can extrapolate that it would take 2 additional weeks until all BrdU$^+$ cells were lost, and hence the entire macrophage population had turned over. We also measured BrdU kinetics in lung and spleen macrophages in the same mice. Here we found a decrease of BrdU$^+$ macrophages from 34.4±2.4 after wash-in to 16.1±0.6% after wash-out in the lung and from 6.7±1.1 to 3.3±0.5 in the spleen. These data are in the range of previously reported values[5], thus validating our experimental set up.

Monocytes contribute sparingly to resident heart macrophages in steady-state

To answer the question if circulating monocytes contribute to the turnover of heart macrophages, we investigated heart macrophage chimerism after putting C57BL/6 CD45.2$^+$ and CD45.1 mice in parabiosis for 6 weeks. During parabiosis, the circulation of two mice links, and circulating cells, including monocytes, mix in the blood of both parabionts. After 6 weeks of parabiosis, the blood chimerism for Ly6C^{high} and Ly6C^{low} monocytes was 22.8±1.1 and 35.3±3.7%, respectively. The chimerism of resident heart macrophages was much lower at 2.7±0.5% (Fig. 3A,B).

While parabiosis provides a convenient tool to study recruitment of cells, it may induce artifacts through pro-inflammatory stimuli. Therefore, to study recruitment in a separate experiment, we adoptively transferred bone marrow hematopoietic stem cells into irradiated recipient mice to i) investigate if monocytes give rise to heart macrophages over a longer period of time, and ii) to enforce higher monocyte blood chimerism than what can be achieved by parabiosis. Recipient ubiquitous GFP$^+$ mice were irradiated (950 cGy) and transplanted with one hundred SLAM HSC harvested from non-GFP CD45.2$^+$ mice. Additionally, we co-transferred GFP$^+$ supportive bone marrow cells which have a limited life span and served the purpose to bridge the recipients' hematopoietic system until the transferred stem cells expand to sustain hematopoiesis, and to respond to any acute radiation-induced heart injury. Eighteen weeks later, we measured the percentages of non-GFP CD45.2$^+$ leukocytes which were derived from donor HSC. While 74.6±8.1% blood monocytes derived from the transferred HSC, only 10.1±1.0% of heart macrophages were descendants of them (Fig. 3C). Together, these data suggest that monocytes only sparingly contribute to resident cardiac macrophages during steady-state.

To assess local proliferation as the source for cell turnover of resident heart macrophages, we used a two-pronged approach. First, mice received one injection of BrdU, followed by

flow cytometric analysis of heart macrophages 2 hours later. We found $BrdU^+$ monocytes in the bone marrow, but they did not yet egress into the blood pool within this short 2 hour time window (Fig. 3D). We found that 2.1±0.3% of heart macrophages stained positive for the BrdU label (Fig. 3D). Second, we performed cell cycle analysis of heart macrophages using Ki67/DAPI flow cytometric staining, which allowed us to distinguish cells in the cell cycle. This experiment provides a snap shot of cells that proliferate while residing in the examined tissue. We detected that 13.7±1.4% of cardiac macrophages were in the cell cycle (Fig. 3E). Together, these data show that cardiac resident macrophages proliferate locally.

Cardiac resident macrophages phagocytose pathogens

To assess if heart macrophages are involved in host defense against pathogens that may enter the myocardium, we injected fluorescent *Staphylococcus aureus* into the left ventricular myocardium. The fluorescence of the probe is only detectable if the bioparticle is activated by exposure to low pH in lysosomes, indicating phagocytosis. We detected fluorescence in 11.4±1.2% of heart macrophages 2 hours after injection of bacteria (Figure 4). These data indicate that macrophages participate in immunosurveillance of the heart.

Monocytes repopulate depleted heart macrophages

We next explored if monocytes contribute to the repopulation of cardiac macrophages following their depletion in $CD11b^{DTR/+}$ mice. To this end, we injected mice with a single dose of diphtheria toxin, thus inducing apoptosis of $CD11b^+$ mononuclear cells in this transgenic mouse. The recovery of blood monocytes and heart macrophages was then monitored by flow cytometry over several days. Ablation of $CD11b^+$ cells drastically reduced the number of circulating monocytes and heart macrophages (Fig. 5A–C). After 3 days, monocytes reappeared in the blood and also invaded the heart, while heart macrophages were still absent. Six days after ablation, the macrophage population recovered (Fig. 5A–C).

To investigate if this recovery was caused by differentiation of recruited monocytes, we studied macrophage recovery in $CD11b^{DTR/+}$/ Ubc^{GFP} parabionts. Two weeks after parabiosis, $CD11b^+$ cells were depleted in the $CD11b^{DTR/+}$ parabiont by injection of diphtheria toxin while GFP^+ donor monocytes of the co-parabiont remained in circulation. On day 6 after depletion, the chimerism for heart macrophages resembled the monocyte chimerism in the blood (Fig. 5D). These data indicate that monocytes repopulate the cardiac macrophage pool after myeloid cell depletion in $CD11b^{DTR/+}$ mice.

Monocytes give rise to infarct macrophages

We next studied the monocyte and macrophage population in mice with coronary ligation, a clinically relevant cardiac injury model. Interestingly, resident heart macrophages were completely lost in 24 hour old infarcts (Fig. 6A) while inflammatory monocytes were entering the infarcted tissue. This observation is unlikely caused by phenotypic cell changes or down-regulation of macrophage markers after ischemia as we used a combination of several leukocyte surface antigens for their detection. Four days after coronary ligation, the macrophage population recovered (Fig. 6A).

The observed time course suggested that after ischemia, infarct resident macrophages arise from monocytes. We directly tested this hypothesis in parabionts with coronary ligation using two different experimental timelines. First, we induced MI in C57BL/6 CD45.2$^+$ mice after these had been in parabiosis with donor GFP$^+$ mice for *two weeks prior to coronary ligation*. Parabiosis continued after MI. On day 4 after MI, the macrophage chimerism in infarct tissue was similar to the monocyte chimerism in blood, indicating that infarct macrophages derived from recruited cells. Even four weeks later, we observed a preserved elevated macrophage chimerism in the infarct (Fig. 6B). Second, we put mice in parabiosis *two weeks after coronary ligation*. Chimerism was measured 4 and 16 weeks later. Interestingly, this alternative timing resulted in lower infarct macrophage chimerism (Fig. 6C), indicating that after the acute phase of monocyte recruitment in the first 2 weeks after MI, the infarct macrophage resident population regains independence from the monocyte blood pool and instead again relies on proliferation as observed in the steady-state.

Macrophages in acutely ischemic myocardium die locally and may emigrate

To explore the mechanism of macrophage reduction in acutely ischemic myocardium, we investigated if cardiac resident macrophages undergo local cell death similar to ischemic myocytes. Indeed, 12 hours after coronary ligation, we found a significantly increased rate of TUNEL$^+$ MAC3$^+$ cells whereas double positive cells were rarely observed in the non-ischemic remote myocardium (Fig. 7A). To begin to understand the kinetics of macrophage death in ischemic tissue, we harvested myocardium 2 hours after coronary ligation for flow cytometric analysis using leukocyte and macrophage surface markers in conjunction with the vital dye propidium iodide (PI) which only enters dead cells. In control hearts, 1.9±0.2% of macrophages stained positive for PI. Interestingly, even after only 2 hours of ischemia, the number of PI$^+$ cells increased 9.5-fold (Fig. 7B).

We had previously observed that infarct macrophages may exit the heart at low numbers[15]. We therefore investigated whether cardiac macrophages which reside in the myocardium prior to coronary artery ligation may exit the acutely ischemic myocardium. To test for macrophage exit, we had to preferentially label cardiac cells but not tissue resident macrophages in likely destination organs. To this end, we stained myocardium in vivo with dioctadecyloxacarbocyanine (DIO). This procedure labeled 12–16% of cardiac resident macrophages (Fig. 8) but failed to stain bone marrow monocytes (Fig. 8G), suggesting that that the myocardial dye injection did not cause unspecific myeloid cell staining in the bone marrow. We next explored whether the the observed disappearance of macrophages after coronary ligation still occurs after intramyocardial dye injection. This was the case, as DIO$^+$ macrophages disappeared at similar rates as observed in mice that did not undergo the labeling procedure (Fig. 8C). Thus, we injected the dye into the myocardium of 8 mice, 4 of which consecutively underwent coronary ligation. 24 hours later, we examined the mediastinal lymph node, the spleen and the bone marrow by FACS. Ischemia did not change the presence of DIO$^+$ macrophages in the mediastinal lymph node (Fig. 8D); however, the percentage of DIO$^+$ macrophages doubled in the spleen and bone marrow of mice that were subjected to myocardial ischemia (Fig. 8E, F).

DISCUSSION

While the role of macrophages in cardiovascular disease is well understood, it was only recently highlighted that there is a sizable tissue resident macrophage population in the normal myocardium at all times[9]. Macrophages were also described in human myocardium by immunoreactive staining, albeit at lower numbers[16]. In contrast to detailed reports on tissue resident macrophages in other organs, the origin and maintenance of cardiac resident macrophages remained unclear until very recently. While our manuscript was in revision, the origins of steady-state cardiac macrophages were described for the first time. The cells arise from embryonic yolk-sac progenitors prior to birth and self-maintain independent of bone marrow-derived monocytes[10]. The results presented here independently confirm that local proliferation dominates supply of local macrophages in the healthy myocardium, as we also found a minimal contribution of blood monocytes to steady-state cardiac resident macrophages.

When the steady-state was perturbed, macrophages mostly derived from blood monocytes. After induction of macrophage apoptosis in CD11b$^{DTR/+}$ mice, monocytes replenished the cardiac macrophage pool. These results correlate well with the observations after clodronate liposome macrophage depletion[10]. While macrophages residing in acute infarcts may also proliferate[17], we found that they overwhelmingly derive from circulating monocytes. These cells are recruited at high levels during the first two weeks after ischemia. If parabiosis was induced 2 weeks after MI, infarct macrophages did not depend on monocyte supply any longer.

Interestingly, we observed a sudden, almost complete disappearance of heart macrophages in the first 24 hours after ischemia in the infarct. Our histologic and FACS data suggest that local cell death, akin to the demise of ischemic myocytes, is a dominant factor in this vanishing act. As soon as 2 hours after ischemia, the number of dead macrophages significantly increased. We also detected an increased number of DIO^{+} macrophages in hematopoietic organs when this cell dye was delivered to the myocardium shortly before coronary artery ligation. It is currently unclear whether these DIO^{+} macrophages actively departed from the ischemic wound, and what the biological significance of this migration may entail. We speculate that these cells could be involved in transfer of danger signals to remote locations.

We currently know very little about cardiac macrophages, which intermingle closely with myocytes, endothelial cells and fibroblasts in the steady-state. The cells have a peculiar spindle-like shape and thus resemble fibroblasts. When viewed together with previous gene expression analyses[9, 10], our histological studies make it unlikely that we confuse these two cardiac residents. The human autopsy data likewise show a spindle shape of CD68^{+} cells. This macrophage-unlike appearance may be caused by the structure of the cardiac tissue. The longitudinal orientation of myocardial fibers possibly dictates the spindle-like macrophage appearance. In day-old infarcts, where the typical longitudinal myocardial structure is lost, macrophages, including GFP^{+} macrophages in the Cx3cr1$^{GFP/+}$ reporter mouse, appear as round cells with dendrites[18], supporting the argument that the tissue environment may form macrophage shapes.

The translational implications of cardiac resident macrophages are foreshadowed by the important tasks of their counterparts in other tissues, where macrophages regulate bone matrix turn-over, dictate the micro-milieu for progenitor and stem cells, instruct the metabolism of resident adipocytes and pursue sentinel functions against invading pathogens[12]. Future work will investigate cardiac-specific tasks of macrophages. Data presented here indicate that the cells participate in immunosurveillance, and may support host defense against pathogens in the heart. Thus, macrophages or their compromised function may be relevant in endocarditis, which is increasingly observed after invasive procedures[19, 20].

A recent report described four subsets of F4/80/MerTK$^+$ resident macrophages with different expression of surface markers Ly6C, MHC-II, and autofluorescence[10]. It remains to be elucidated if these macrophage subsets pursue distinct functions. It is clear, however, that a precise understanding of their tasks is a prerequisite for therapeutic targeting of these cells. Overabundance of inflammatory macrophages in the infarct compromises repair and promotes heart failure[8], rendering these cells a potential drug target. The selective monocyte contribution to infarct macrophages may thus offer the opportunity to target infarct macrophages via interfering with monocyte recruitment, for instance with in vivo RNAi[21], while sparing resident macrophages in the non-ischemic myocardium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

BrdU	bromodeaoxyuridine
DIO	dioctadecyloxacarbocyanine
DT	diphteria toxin
FACS	fluorescence activated cell sorting
GFP	green fluorescent protein
HSC	hematopoietic stem cells
MI	myocardial infarction
PI	propidium iodide

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Novelty and Significance

What Is Known?

- Macrophages are innate immune cells residing in most tissues, including the steady state myocardium and the acutely infarcted heart.
- Many inflammatory macrophages derive from circulating monocytes while some resident macrophages, for instance in the brain or lung, do not.

What New Information Does This Article Contribute?

- In the steady state, cardiac resident macrophages mostly derive from local proliferation and self-maintain independently of circulating monocytes.
- In contrast, bone marrow derived monocytes give rise to macrophages in acute infarcts and after depletion.
- Cardiac resident macrophages participate in immune surveillance.

Macrophages are frequent innate immune cells with important general and specialized functions in all major organs. For instance, macrophages provide a first line of defense in wound healing and against infection. However, the cells also give rise to pathology, including in atherosclerotic plaque and after myocardial infarction. Thus, they represent emerging therapeutic targets that have to be addressed with utmost care to avoid collateral damage. Recent data suggest that there are numerous cardiac macrophages in healthy and diseased hearts; however, their supply, a potential therapeutic target, was incompletely understood. The data presented here suggest a dichotomy: macrophages in healthy myocardium derive from local proliferation whereas injury to the heart triggers a monocyte influx. These monocytes then differentiate into macrophages, suggesting that targeting cell recruitment or production in hematopoietic tissues is a potential therapeutic strategy to reduce inflammation in the heart without compromising resident macrophages. In addition, we report that cardiac macrophages react to myocardial ischemia with tissue exit and, dominantly, local death.

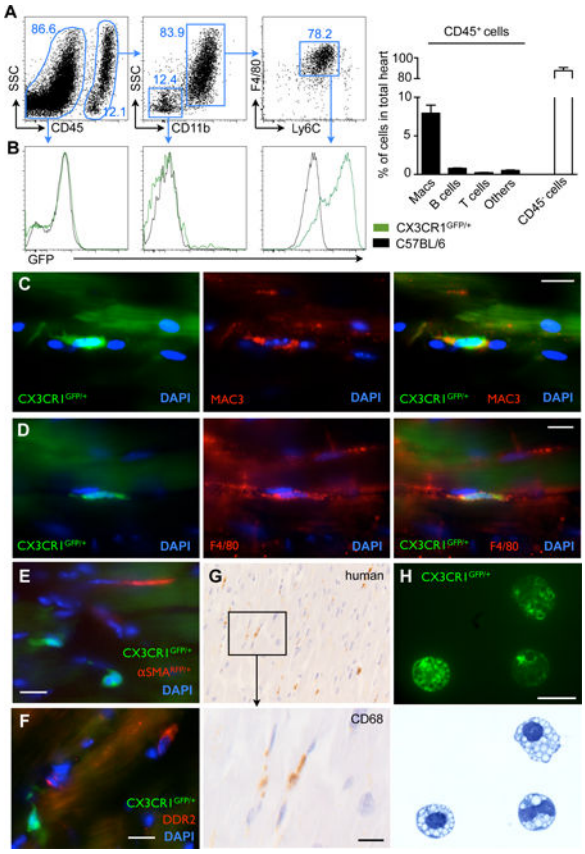

Figure 1. Resident cardiac macrophages in the healthy heart

A, Gating strategy for identification of CD45^{-} non-leukocyte cells, lymphocytes (identified as CD45^{+}, CD11b^{-} and SSC^{low}) and heart macrophages (identified as CD45^{high} F4/80^{high} Ly6C^{low}) by flow cytometry. Frequencies within the entire heart are provided as mean ±SEM. **B**, GFP expression in Cx3cr1$^{GFP/+}$ mice in different cell types, compared to C57BL/6 mice. **C**, Immunofluorescence microscopy of healthy heart tissue showing co-staining of MAC3 or F4/80 (**D**) with nuclear staining (DAPI) and the Cx3cr1$^{GFP/+}$ reporter (right). **E**, Immunofluorescence microscopy of healthy myocardium in a Cx3cr1$^{GFP/+}$ αSMA$^{RFP/+}$ dual reporter mouse. **F**, Immunofluorescence microscopy of healthy myocardium in a Cx3cr1$^{GFP/+}$ reporter mouse, stained for the fibroblast reporter DDR2. **F**, Immunohistochemical staining for CD68 in human myocardium. Scale bars indicates 5μm.

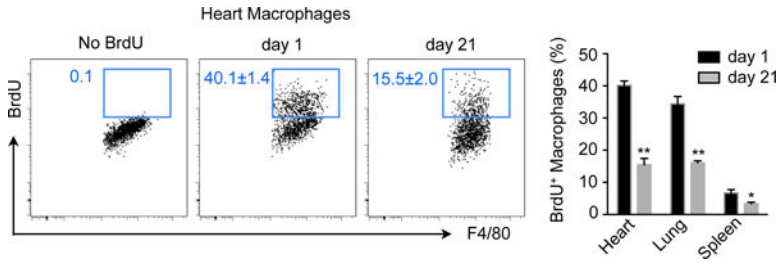

Figure 2. Resident cardiac macrophages turn over slowly in the steady-state

Mice were given daily BrdU injections for 4 weeks. BrdU incorporation in tissue resident macrophages (heart, lung and spleen) was measured immediately and 21 days after the last BrdU injection (n=6 per group, mean±SEM, *p<0.05 versus day 1, **p<0.01). One control mouse per cohort was not injected with BrdU to serve as a staining control (no BrdU).

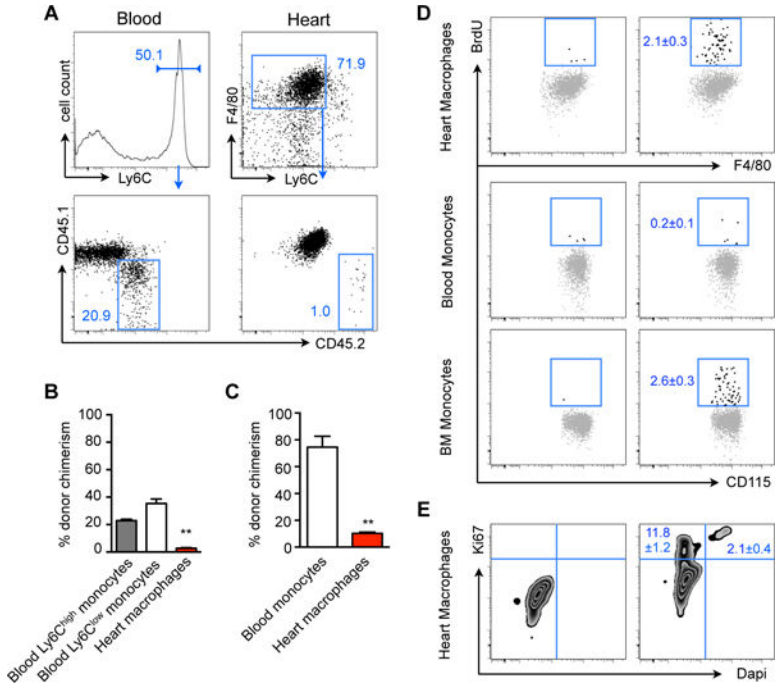

Figure 3. Resident cardiac macrophages self-maintain through local proliferation

A, C57BL/6 CD45.2^{+} and CD45.1 mice were put in parabiosis for 6 weeks. Plots show representative data for chimerism of parabiont-derived cells from blood monocytes and heart macrophages. Upper dot plots are gated on CD11b^{+} CD115^{+} cells. **B**, Bar graph depicts relation of donor-derived chimerism (n=8, mean±SEM). **C**, Recipient Ubc^{GFP+} mice were lethally irradiated and transplanted with 100 Lin^{neg} cKit^{+} Sca-1^{+} CD48^{-} CD150^{+} hematopoietic stem cells obtained from CD45.2^{+} mice. Bar graph displays donor-derived CD45.2^{+} cells among blood monocytes and heart macrophages 18 weeks later (n=8, mean ±SEM). **D**, Dot plots show BrdU^{+} incorporation in heart macrophages 2 hours after one dose of BrdU (upper panel) compared to the blood monocytes (middle panel) and bone marrow monocytes (lower panel, n=4 each, mean±SEM). A mouse without BrdU injection served as staining control (left panels). **E**, Cell cycle analysis with Ki67/DAPI staining on heart macrophages (left: isotype control, right: Ki67/DAPI, n=5, mean±SEM).

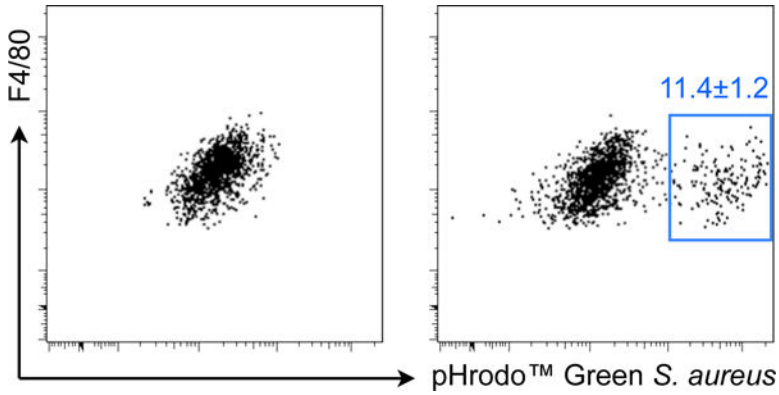

Figure 4. Macrophages phagocytose Staphylococcus aureus

Mice were injected with fluorescent staphylococcus aureus bioparticles into the left ventricular myocardium. Two hours later, fluorescence within cardiac macrophages was analyzed by flow cytometry (right panel, n=5, mean±SEM). One mouse which was not injected served as staining control (left panel). Gated on $CD45^+$ $Lineage^-$ $CD11b^+$ cardiac cells.

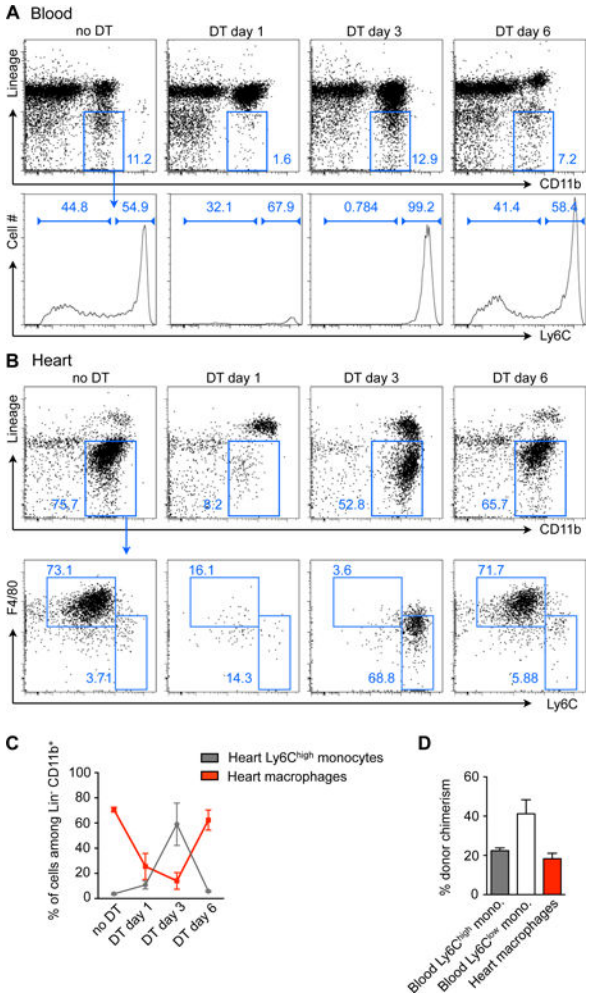

Figure 5. Monocytes contribute to macrophage recovery following induction of macrophage apoptosis

$CD11b^{DTR/+}$ mice were injected with a single dose of diphtheria toxin (DT, n=3 per time point). FACS plots show depletion and recovery in blood (**A**) and the heart (**B**). **C**, Percentages of $F4/80^{low}$ $Ly6C^{high}$ monocytes and $F4/80^{high}$ $Ly6C^{low}$ macrophages in the heart after one DT injection, gated on $CD45^{+}$ Lin^{-} $CD11b^{+}$ cells. **D**, Macrophages were depleted in $CD11b^{DTR/+}$ mice 2 weeks after establishing parabiosis with Ubc^{GFP+} mice. Percentages of donor GFP^{+} cells in peripheral blood monocytes and cardiac macrophages were assessed in parabionts after 6 days (n=4).

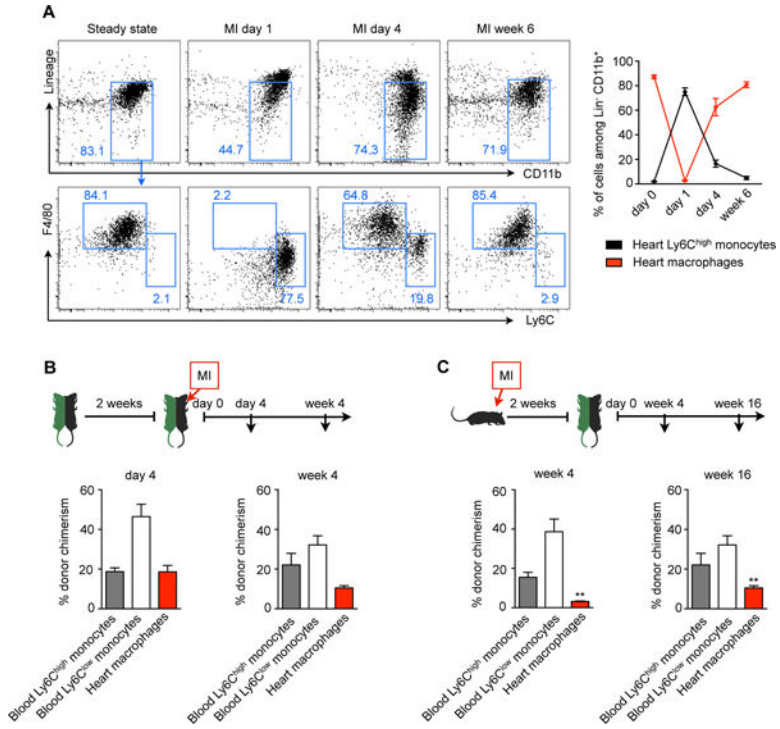

Figure 6. Monocytes repopulate macrophages in ischemic tissue

A, Dot plots show kinetics of F4/80^{low} Ly6C^{high} monocytes and F4/80^{high} Ly6C^{low} macrophages in the heart after MI. The graph indicates percentages of Lin^{-} CD11b^{+} F4/80^{low} Ly6C^{high} monocytes and F4/80^{high} Ly6C^{low} macrophages in the MI (n=3–4 per time point). **B**, MI was induced in C57BL/6 CD45.2^{+} mice after parabiosis with Ubc^{GFP+} mice. Percentages of donor GFP^{+} cells in peripheral blood monocyte subsets and heart macrophages were assessed in parabionts 4 days (n=6 pairs) and 1 month after MI (n=5 pairs). **C**, C57BL/6 CD45.2^{+} and Ubc^{GFP+} mice were put in parabiosis 2 weeks after MI. Percentages of GFP^{+} cells among peripheral blood monocytes and heart macrophages were assessed in parabionts after 4 weeks (n=8) and 16 weeks (n=4 mean±SEM, **p<0.01 versus blood Ly6C^{high} monocytes).

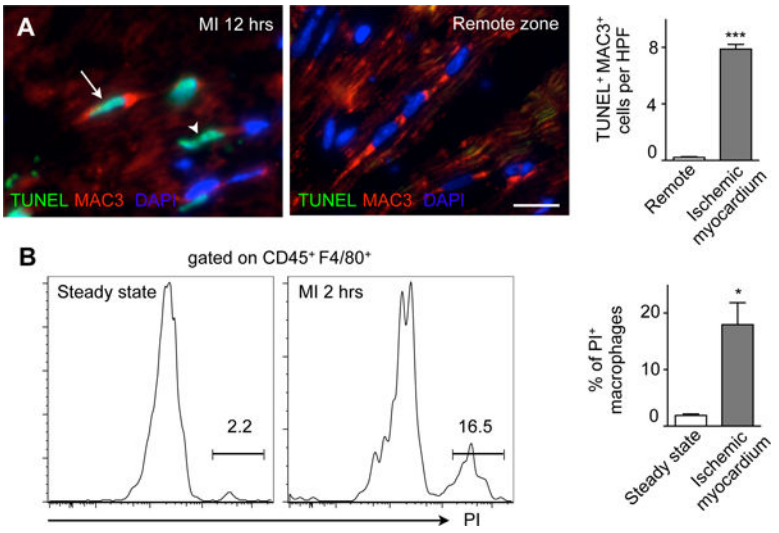

Figure 7. Macrophage death

A, TUNEL^{+} MAC3^{+} macrophages (arrow) were observed in ischemic myocardium 12 hours after MI but not in the remote zone (n=3 mice, 10 fields of view per mouse, mean±SEM, ***p<0.001). Arrow head: TUNEL^{+} MAC3^{-} cell. Scale bar indicates 5μm. **B**, FACS analysis of myocardium 2 hours after MI, compared to non-MI controls. Representative histograms show the increase in propidium iodide (PI) positive, i.e. dead macrophages. Prior gates include CD45^{+} leukocytes and F4/80^{+} macrophages (n=2 controls, 4 mice with MI, mean±SEM, *p<0.05).

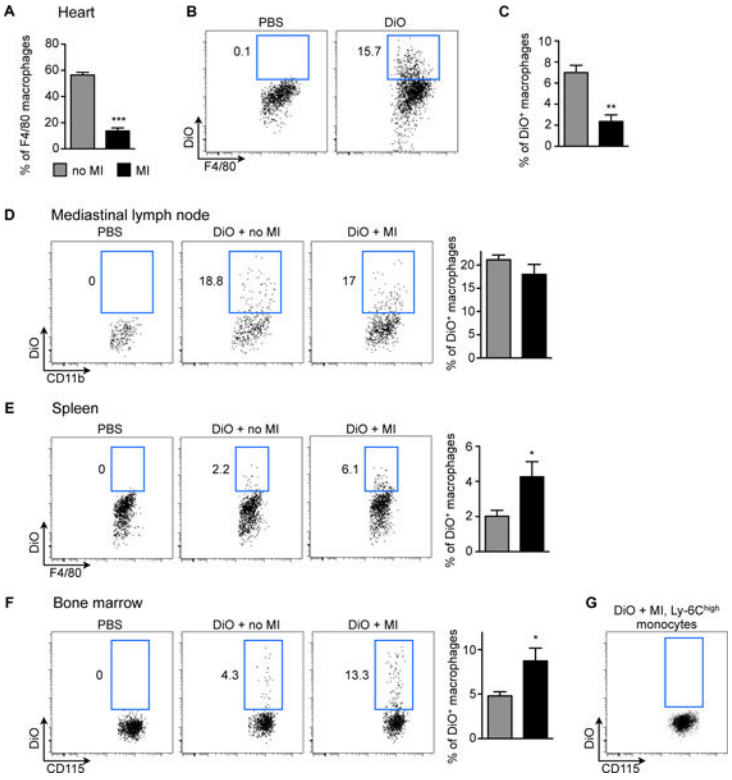

Figure 8. Macrophage exit

DiO lipophilic membrane dye was injected into the myocardium of C57BL/6 mice prior to coronary ligation. Mice injected with the dye but without MI or mice injected with PBS were used as controls. **A**, Twenty four hours later, reduced frequencies of cardiac macrophages are observed in mice with MI. The procedure resulted in DiO^+ macrophages (**B**) which also were reduced in frequency after MI (**C**). MI did not change the frequency of DiO^+ macrophages in mediastinal lymph nodes (**D**) but in spleen (**E**) and in the bone marrow (**F**). (**G**) The dye was not found in bone marrow $Ly6C^{high}$ monocytes, cells that served as controls as they are not observed in the steady state myocardium (n=4 mean ±SEM, *p<0.05 and **p<0.01).