SUPPLEMENTAL MATERIAL

Proliferation and recruitment contribute to myocardial macrophage expansion in chronic heart failure

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Detailed Methods

Flow cytometry. Blood for flow cytometric analysis was collected by cardiac puncture using a 50 mM EDTA (Ethylene Diamine Tetra Acetic Acid) solution (Sigma Aldrich) as an anticoagulant. Erythrocytes were lysed using a red blood cell lysis buffer (BioLegend). Hearts were extensively flushed with PBS and then excised. Remote myocardium was separated from infarct and border zone using a dissection microscope, minced with scissors and digested in collagenase I (450 U/ml), collagenase XI (125 U/ml), DNase I (60 U/ml) and hyaluronidase (60 U/ml) (Sigma-Aldrich) at 37^{o} C at 750 rpm for 1 hour. Hearts were subsequently homogenized through a 40-µm nylon mesh. Spleens were excised and homogenized through a 40-µm cell strainer. Bone marrow was flushed out from bones and homogenized through 40-µm cell strainers. Total viable cell numbers were obtained using Trypan blue (Cellgro, Mediatech, Inc.). For a myeloid cell staining, cells were first labeled with mouse hematopoietic lineage markers including anti-mouse antibodies directed against B220 (BD Biosciences, clone RA3-6B2), CD90 (BioLegend, clone 53-2.1), CD49b (BD Biosciences, clone DX5), Ly-6G (BD Biosciences, clone 1A8), NK1.1 (BioLegend, clone PK136) and Ter-119 (BD Biosciences, clone TER-119). This was followed by a second staining including CD45.2 (BD Biosciences, clone 104), CD11b (BD Biosciences, clone M1/70), CD115 (BioLegend, AFS98), CD11c (BD Biosciences, clone HL3), F4/80 (BioLegend, clone BM8), MhcII (BioLegend, clone M5/114.15.2), Ccr2 (R&D, clone 475301), and Ly6C (BD Biosciences, clone AL-21). Neutrophils were identified as (CD90/B220/CD49b/NK1.1/Ter119)^{low}, (CD45.2/CD11b)^{high}, CD115$^{low/int}$, Ly6G^{high}. Monocytes were identified as (CD90/B220/CD49b/NK1.1/Ter119)^{low}, CD11b^{high}, (F4/80/CD11c)^{low}, Ly-6C$^{high/low}$ or (CD45.2/CD11b)^{high}, Ly6G^{low}, CD115^{high}, Ly-6C$^{high/low}$. Macrophages were identified as (CD90/B220/CD49b/NK1.1/Ter119)^{low}, CD11b^{high}, Ly6C$^{low/int}$, Ly6G^{low}, F4/80^{high}. For a hematopoietic stem/progenitor cell staining, cell suspensions were labeled with biotin-conjugated anti-mouse antibodies (lineage for hematopoietic stem/progenitor staining) directed against B220 (eBioscience, clone RA3-6B2), CD11b (eBioscience, clone M1/70), CD11c (eBioscience, clone N418), NK1.1 (eBioscience, clone PK136), Ter-119 (eBioscience, clone TER-119), Gr-1 (eBioscience, clone RB6-8C5), CD8a (eBioscience, clone 53-6.7), CD4 (eBioscience, clone GK1.5) and Il7rα (eBioscience, clone A7R34) followed by a labeling with an anti-biotin pacific orange-conjugated streptavidin antibody. Cell suspensions were then stained with antibodies directed against c-kit (BD Bioscience, clone 2B8), Sca-1 (eBioscience, clone D7) and SLAM markers CD48 (eBioscience, clone HM48-1) and CD150 (Biolegend, clone TC15-12F12.2). Hematopoietic Lin^{-} Sca-1^{+} c-kit^{+} (LSK) were identified as (B220/CD11b/CD11c/NK1.1/Ter-119/Gr-1/CD8a/CD4/Il7rα)^{low} c-kit^{high} Sca-1^{high}, hematopoietic stem cells (HSC) as (B220/CD11b/CD11c/NK1.1/Ter-119/Gr-1/CD8a/CD4/Il7rα)^{low} c-kit^{high} Sca-1^{high} CD48^{low} CD150^{high}. Data were recorded with an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo version 9 (Tree Star, Inc.). For cell cycle analysis**,** cells were fixed and permeabilized after surface staining using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Intracellular staining was performed by targeting nuclear antigen Ki67 (eBioscience, clone SolA15) and DNA (4,6-diamidino-2-phenylindole, DAPI, FxCycle Violet Stain, Life Technologies).

Cell sorting. Before sorting of cardiac macrophages, we stained samples with biotin conjugated CD11b (BD Biosciences, clone M1/70) and subsequently with streptavidin microbeads (Miltenyi), performed a positive selection using magnetic-activated cell sorting (MACS, QuadroMACS Separator and LS Columns, Miltenyi), followed by sorting on a FACSAria II (BD Biosystems).

Macrophage calculation from parabiosis experiments. For calculating the contribution of recruitment to expansion of remote macrophage from the non-ischemic zone, we used data obtained in the parabiosis experiment shown in Figure 2D. We first assessed the chimerism of GFP^{+} cells in blood and heart in the infarcted wild type parabiont (Figure 2E). We then

calculated recruitment by normalizing heart to blood chimerism for each mouse. For instance, if blood chimerism was 23% and heart chimerism 4.2%, recruitment equaled 18.3% after normalization to blood: 100% / 23% x 4.2% = 18.3% (Figure 2F). Four weeks after MI, we counted 4,884 macrophages/mg myocardium by flow cytometry, increased from 2,715 in steady-state (Figure 1C). Thus, an additional 2,169 macrophages accumulated per mg failing myocardium. The parabiosis experiment, and above calculation returned that on average, 14.5% of the total 4,884 macrophages originated from recruitment (on average 708 GFP^+ macrophages/mg remote myocardium in non-GFP parabiont's infarcted heart, experiment outlined in Figure 2D). We then normalized these 708 macrophages to the mean number of macrophages added by heart failure: 100% / 2,169 x 708 = 32.6%, yielding that approximately one third of the macrophage increase was driven by recruitment (Figure 2G).

Cell culture of murine macrophages. We euthanized wild-type mice and flushed the peritoneal cavity with 5 mL PBS containing 3% FBS and 0.4% EDTA and recovered the peritoneal fluid. We then stained cells with anti-mouse PE-conjugated antibodies directed against B220 (BD Biosciences, clone RA3-6B2), CD90 (BioLegend, clone 53-2.1), CD49b (BD Biosciences, clone DX5), NK1.1 (BioLegend, clone PK136) and Ter-119 (BD Biosciences, clone TER-119) and subsequently with anti-PE microbeads (Miltenyi). We next performed a negative selection using magnetic-activated cell sorting (MACS, QuadroMACS Separator and LD Columns, Milteni) according to the manufacturer's instructions. After manually counting cell numbers, primary murine peritoneal macrophages were plated at a density of $1x10^6$ cells/dish in 13 mL of cell culture medium (DMEM containing 14% FBS, 2% Pen/Strep, 2% MEM, and 0.00086% β-ME, Corning and Sigma). 48h after plating, we exchanged the cell culture medium and added 1 mg of BrdU (bromodeoxyuridine, BD Bioscience) to each dish where appropriate, and then initiated stretching as described below. 24h later, cells were harvested by scraping the dishes. We then performed cell surface staining with anti-mouse PE-conjugated antibodies directed against B220 (BD Biosciences, clone RA3-6B2), CD90 (BioLegend, clone 53-2.1), CD49b (BD Biosciences, clone DX5), Ly-6G (BD Biosciences, clone 1A8), NK1.1 (BioLegend, clone PK136) and Ter-119 (BD Biosciences, clone TER-119). This analysis was followed by a second staining covering CD45.2 (BD Biosciences, clone 104), CD11b (BD Biosciences, clone M1/70), F4/80 (BioLegend, clone BM8), and Ly6C (BD Biosciences, clone AL-21). Intracellular staining for the nuclear antigen Ki67 (eBioscience, clone SolA15) or BrdU (BrdU flow kits, BD Bioscience) was carried out as described previously to assess proliferation[1].

ERK1/2 ELISA. For the semi-quantitative measurements of phosphoERK1/2 (pT202/Y204) and total ERK1/2 an ELISA kit was used on lysed stretched and non-stretched cultured murine peritoneal macrophages in accordance to the instruction manual (ERK1/2 (pT202/Y204) + Total ERK1/2 SimpleStep ELISA Kit, Abcam).

In-dish-imaging. After stretching, we added F4/80 (BioLegend, clone BM8) directly to the culture medium. After 10 min of staining, cells were fixed and permeabilized using the Foxp3/ Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions and then stained with Ki67 (eBioscience, clone SolA15) and 4,6-diamidino-2-phenylindole (DAPI, FxCycle Violet Stain, Life Technologies). In-dish-imaging used a confocal microscope (Olympus IV100).

Mek1/2 inhibition. In cell culture experiments, we used the Mek1/2 inhibitor PD98059 (Calbiochem). We seeded primary peritoneal macrophages and replaced the cell culture medium 48h thereafter with PBS containing either 50 mmol/L PD98059 (dissolved in DMSO (dimethyl sulfoxide, Sigma-Aldrich)) or vehicle alone (DMSO). After 1h of incubation, we again replaced the PBS with cell culture medium and initiated stretching for 24h. For in vivo studies, we used PD0325901 (Selleckchem), a second-generation small-molecule inhibitor selective for Mek1/2. When we prepared PD0325901 for injections, we diluted it with Kolliphor EL (Sigma-

Aldrich) and PBS. We injected 10 mg/kg bodyweight PD0325901 i.p. once daily starting 7d after coronary ligation for three weeks. Control mice received vehicle (DMSO, Kolliphor EL, and PBS).

Cell culture of human macrophages. Peripheral blood monocytes were obtained from buffy coats from de-identified healthy donors. Monocytes were isolated by density gradient centrifugation with Lymphocyte Separation Medium 1.077 (Lonza)[2]. Adherent monocytes were differentiated into macrophages for 10 days in RPMI 1640 (Gibco) with L-glutamine and 5% human serum. Macrophages were rinsed once with sterile Hanks' buffered saline solution (HBSS, Gibco) and harvested with a cell scraper, then seeded at a density of $1x10^6$ on a silicon elastomer membrane assembled for mechanical strain. Surface staining relied on anti-human CX3CR1 (BioLegend, clone 2A9-1), CD14 (BioLegend, clone HCD14), CD11b (BioLegend, clone ICRF44) and CD68 (BioLegend Y1/82A).

Magnetic resonance imaging (MRI). MRI was carried out on 4 weeks after permanent coronary ligation as described perviously[1]. Using a 7 Tesla horizontal bore Pharmascan (Bruker) and a custom-built mouse cardiac coil (Rapid Biomedical), we obtained cine images of the left ventricular short axis which we analyzed using the software Segment (http://segment.heiberg.se). Four weeks after myocardial infarction, scar tissue was identified as akinetic myocardial segments with relevant thinning of the myocardial wall. Scar and remote volumes in each myocardial slice were quantified using OsiriX© software. 7-8 myocardial slice were analyzed per heart.

CFU-assay. A semi-solid cell culture medium (Methocult M3434, Stem Cell Technology) was used for performing colony forming unit (CFU) assays as recommended by the manufacturer. We flushed bones with Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with 2% fetal calf serum. We plated $2x10^4$ bone marrow cells in duplicates. After incubating for 12 days, we counted colonies on an inverted microscope.

Quantitative real-time PCR. RNA was extracted from either bone marrow or hearts using the RNeasy Mini Kit (Qiagen) or from FACS-isolated macrophages using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) according to the manufacturers' protocol. mRNA was transcribed to complimentary DNA (cDNA) with the high capacity RNA to cDNA kit (Applied Biosystems). Quantitative PCR used Taqman probes (Applied Biosystems). Results were expressed by delta delta Ct values normalized to *Gapdh*.

Noradrenaline high sensitivity ELISA. For measurements of noradrenaline in the bone marrow, a 2–CAT (A–N) Research ELISA (kit EA633/96, Diagnostika GmbH) was carried out according to the manufacturer's instructions. One femur was snap-frozen and immediately homogenized in a catecholamine stabilizing solution (pH adjusted to 7.5 using sodium hydroxide (1 N)) containing sodium metabisulfite (4 mM), EDTA (1 mM) and hydrochloric acid (0.01 N).

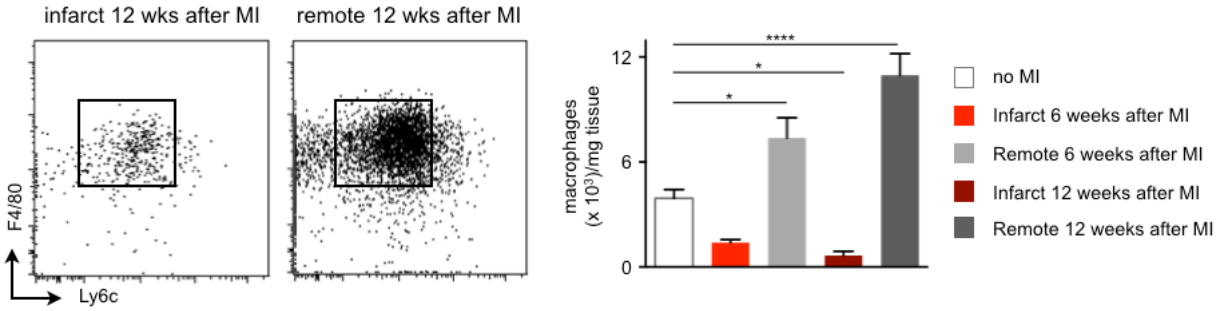
Histologic analysis of murine hearts. Hearts were flushed thoroughly with PBS and then harvested. We embedded hearts in O.C.T. compound (Sakura Finetek) and snap-froze them in a 2-methylbutane bath cooled with dry ice. For immunohistochemistry, 6 µm frozen sections were cut and stained using antibodies targeting α-smooth muscle actin (αSma, Abcam, ab5694), collagen I (Abcam, ab21286), and CD31 (BD Biosciences, clone MEC13.3). We then applied the respective biotinylated secondary antibodies and used a the VECTASTAIN ABC kit (Vector Laboratories, Inc.) and an AEC substrate (Dako) for color development. All sections were counterstained with Harris hematoxylin. We scanned slides and quantified the positive areas (five high-power fields per section and per animal) using iVision (version 4.5, Biovis).

Histologic analysis of human hearts. Human myocardial specimens were obtained from patients either at the time of a left ventricular assist device (LVAD) placement (ischemic cardiomyopathy samples) or from donors not suitable for transplantation (donor specimens). Ischemic cardiomyopathy tissue specimens consisted of transmural left ventricular myocardium harvested from the apex in patients with inferior and lateral infarcts. The presence of remote myocardium was confirmed by histological analysis. Donor tissue specimens consisted of transmural apical LV myocardium harvested from donors with normal left ventricular function as documented by echocardiography. Following procurement samples were fixed in 10% neutral buffer formalin, embedded in paraffin, and sectioned. For each specimen, at least three 4 μm sections were stained with antibodies specific for human anti-CD68 (Thermo, 1:2000) and Ki67 (Abcam, 1:1000). Antibody detection was performed using a tryamide-based signal amplification system per manufacturer protocol (Perkin Elmer). Samples were counterstained with DAPI and imaged on a Zeiss confocal microscope. Quantification of CD68 and Ki67 staining was then performed in blinded fashion. Human studies were reviewed, approved, and conducted in accordance with the Human Studies Committee Intuitional Review Board at Washington University (IRB protocol 201305086).

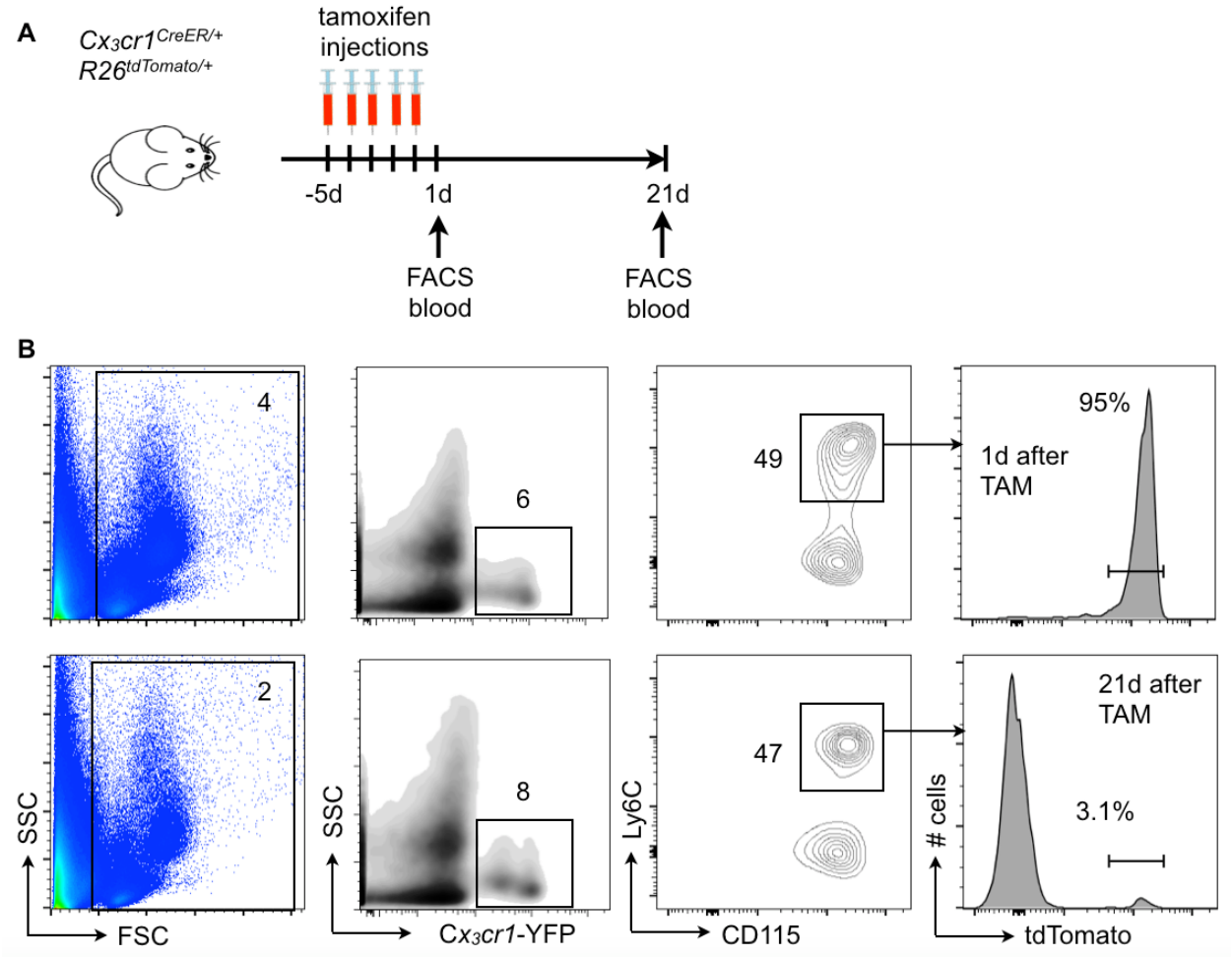
siRNA formulation into 7C1 nanoparticles. Purified 7C1 nanoparticles were synthesized and formulated as previously described[3]. Polyethyleneimine with a molecular weight of 600 ($PEI_{600,}$ Sigma Aldrich) was combined with 200 proof anhydrous ethanol (Koptec) and an epoxide-terminated C_{15} lipid at a lipid:PEI molar ratio equal to 14:1. The mixture was heated at 90˚C for 48 hours before purification was performed with a silica column. To formulate nanoparticles, purified 7C1 was combined with 200 proof ethanol and $C_{14}PEG_{2000}$ (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], Avanti Polar Lipids) at a 7C1:$C_{14}PEG_{2000}$ molar ratio equal to 4:1 in a glass syringe. siRNA was dissolved in pH 3 10 mM citrate solution (Teknova) in a separate syringe. The two syringes were connected to a syringe pump and the fluid was pushed through a microfluidic device as previously described. The resulting nanoparticles were dialyzed in 1x PBS and filtered through a 0.22 μm sieve before their size was characterized.

Nanoparticle and siRNA dosing. Nanoparticles were injected intravenously via tail vein in a volume of 10 μL/g body weight. Particles contained either 3.0 mg/kg of siRNA targeting luciferase (termed siCtrl, non-targeting control siRNA) or 3.0 mg/kg of a combination of five siRNAs encapsulated in the same particle targeting the leukocyte adhesion molecules Icam1, Icam2, Vcam1, E-selectin, and P-selectin (termed siCAM[5]). The selection of siRNA sequences was described previously[4]. The following sequences were used: Icam1, sense CUUUCCUUUGAAUCAAUAA, antisense UUAUUGAUUCAAAGGAAAG; Icam2, sense AGGACGGUCUCAACUUUU, antisense GAAAAGUGAGACCGUCCU; Vcam1, sense ACUGGGUUGACUUUCAGGU, antisense ACCUGAAAGUCAACCCAGU; Eselectin, sense GCCAAAGCCUUCAAUCGUU, antisense AACGAUUGAAGGCUUUGGC; Pselectin, sense GCACAAAUGUAUGUCGUAU, antisense AUACGACAUACAUUUGUGC.

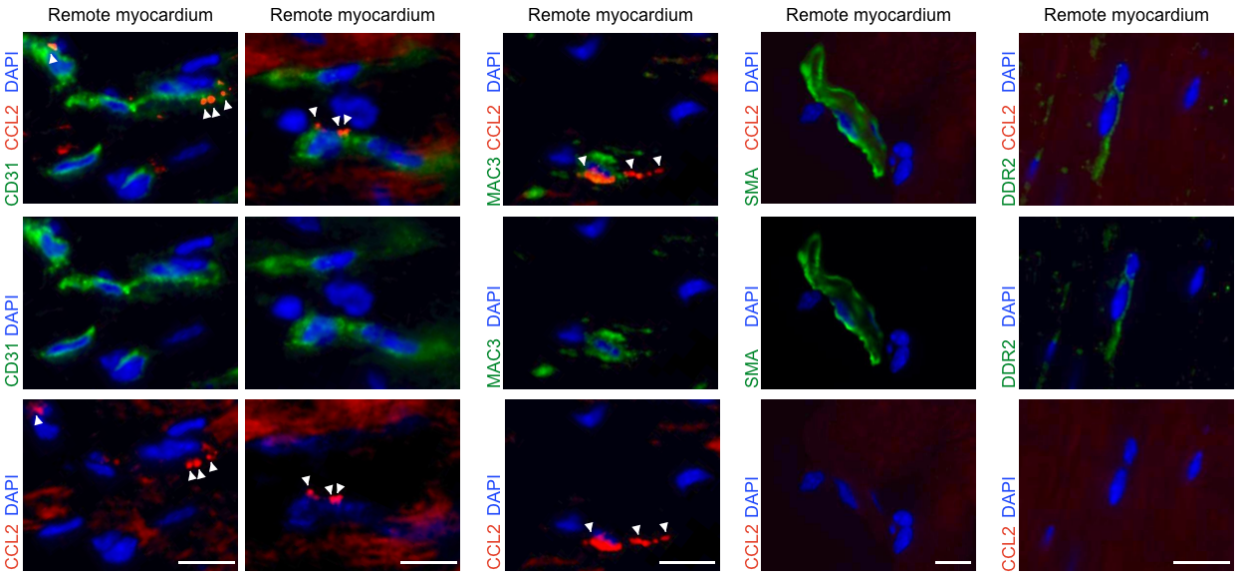
Supplemental Figures and Figure Legends



Supplemental Figure I.
Gating and quantification of cardiac macrophages in steady-state versus HFrEF (6 and 12 weeks after MI, remote area), n=4-5 WT mice per group, mean±SEM, *p<0.05, ****p<0.0001).

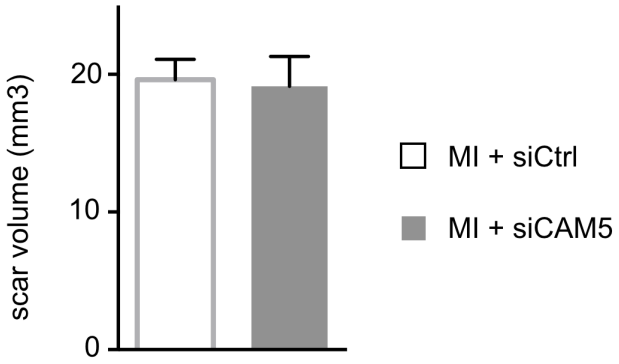


Supplemental Figure II.
Timeline for red fluorescence signal in blood monocytes in fate mapping.
A, Experimental design.
B, Representative gating and assessment of tdTomato red fluorescence signal in blood Ly6C^{high} monocytes after a tamoxifen pulse, n=3 mice per group.



Supplemental Figure III.

Cellular source of Ccl2. Representative immunofluorescence histology of remote myocardium 4 weeks after MI (n=5 mice per group). Co-localization of Ccl2 with endothelial cells (CD31), macrophages (Mac3), fibroblasts (DDR2), and myofibroblasts (SMA). Scale bar, 10 μm.



Supplemental Figure IV.
Evaluation of scar area day 28 after MI by cardiac MRI. Treatment with either siCtrl or siCAM5 was initiated 7d after coronary ligation (n=9-11 per group, mean±SD).

Supplemental References

1. Sager HB, Heidt T, Hulsmans M, Dutta P, Courties G, Sebas M, Wojtkiewicz GR, Tricot B, Iwamoto Y, Sun Y, Weissleder R, Libby P, Swirski FK, Nahrendorf M. Targeting Interleukin-1beta Reduces Leukocyte Production After Acute Myocardial Infarction. *Circulation*. 2015;132(20):1880-90.
2. Isoda K, Folco E, Marwali MR, Ohsuzu F, Libby P. Glycated LDL increases monocyte CC chemokine receptor 2 expression and monocyte chemoattractant protein-1-mediated chemotaxis. *Atherosclerosis*. 2008;198:307-312.
3. Dahlman JE, Barnes C, Khan O et al. In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nat Nanotechnol*. 2014
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