ONLINE SUPPLEMENT

The macrophage mineralocorticoid receptor is a pleiotropic modulator of myocardial infarct healing

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MATERIAL AND METHODS

Myocardial infarction

Myocardial infarction was induced by permanent left coronary artery ligation in age- and gender-matched mice. Briefly, mice were anesthetized with 1.8% isoflurane in a 100% oxygen mix, intubated, and ventilated using a ventilator (MINIVENT mouse ventilator model 845) with the tidal volume adjusted based on body weigh (10µL/g BW). Buprenorphine (0.1 mg/kg BW) was intraperitoneally administered for postoperative pain relief. The thoracic cavity was opened through an incision between the 3rd and 4th intercostal space. A rib spreader was introduced to allow for visualization of the heart. The left coronary artery was ligated with a 6-0 silk suture just below the left auricular level. In sham-operated mice the suture passing around the left anterior descending coronary artery was not tied.[1, 2]

Hemodynamic and volume measurements

Hemodynamic and volume measurements were performed 7 days after coronary artery ligation, under light isoflurane anesthesia and spontaneous respiration, using a conductance catheter (SPR-839, Millar Instruments) connected to a data acquisition system (ADInstruments PowerLab, Chart 5). Pvan software (Millar) was used to analyze all pressure-volume loop data recorded at steady-state and during injection of hypertonic saline for the calibration of parallel conductance volume (V_p). Left ventricular (LV) volume was calculated for each mouse from conductance volume corrected by the relative V_p.[1, 2]

Infarct Size, scar collagen content, collagen fiber architecture.

The heart was arrested in diastole by potassium chloride. 5µm thin sections were serially cut from apex to base and stained with 0.1% sirius red F3B in saturated picric acid. Four lengths were derived from each digital image of the sections, including epicardial and endocardial infarct lengths and epicardial and endocardial circumferences, using Sigma Scan Pro 5.0 image analysis software (Systat Software Inc). Epicardial infarct ratio was obtained by dividing epicardial infarct length by epicardial circumference. Endocardial infarct ratio was calculated similarly. Infarct size derived from this approach was calculated as [(epicardial infarct ratio+endocardial infarct ratio)/2] x 100.[1, 2] To assess scar collagen content and collagen fiber architecture LV sections were examined using a Nikon ECLIPSE 50i microscope equipped with filters to provide circularly polarized illumination. Tissue images were recorded with a cooled digital camera (DS-5Mc, Nikon) and analyzed using SigmaScan Pro 5.0 image analysis software (Systat Software Inc). Collagen content was expressed as a percentage of the area of each image.[1, 2] The quality of collagen fibers was derived from the hue component of each color image[3] and calculated as: ratio of the thick tightly assembled mature collagen fibers as orange-red birefringent to the thin loosely packed immature fibers as yellow-green birefringent (orange-red/yellow-green ratio). Alignment and straightness of the fibers were determined using CurveAlign V4.0 Beta, a MATHLAB based software platform with fraction of kept cuvelets set to 0.06 and all other settings as default.[4]

Immunofluorescence

For immunofluorescence double staining, LV cryostat sections were blocked with 2% donkey serum and incubated overnight with the primary antibody against CD31 (MCA2388, Bio-Rad) followed by incubation with Cy™3 AffiniPure Donkey Anti-Rat (712-165-153, Jackson Immuno Research). Subsequently, sections were stained using the Mouse on Mouse (M.O.M.™) Kit (BMK-2202, Vector Laboratories) and FITC-labeled anti-α-SMA (F3777, Sigma). For CD68-immunofluorescence sections were stained with Alexa Fluor 594–labeled

anti-CD68 (137020; BioLegend). Nuclei were stained with NucBlue Live Cell Stain (R37605, Molecular Probes™).

Isolation of cardiac macrophages and fibroblasts

A method aimed at preserving cell viability, gene expression profiles, antigens and morphology was developed to obtain a single cell suspension at steady state and after myocardial infarction.[1, 5] In brief, the hearts were perfused for 6 minutes with the Perfusion Buffer (113mM NaCl, 4.7mM KCl, 0.6mM KH_2PO_4, 0.6mM Na_2HPO_4), 1.2mM $MgSO_4$, 12mM $NaHCO_3$, 10mM $KHCO_3$, 10mM HEPES, 30mM Taurine, 5.5mM glucose, 10mM 2,3-Butanedione monoxime), and subsequently digested for 8 minutes with the Digestion Buffer (0.2mg/mL Liberase™ Roche Diagnostics; and 400µM calcium chloride in perfusion buffer), using a modified Langendorff perfusion system. The ischemic area und surviving myocardium were separated using a dissecting microscope. Subsequently, the heart tissue was smoothly pipetted through a sterile low waste syringe several times in order to obtain a cell suspension in 5 mL of Stop Buffer (perfusion buffer supplemented with 10% (v/v) HI-FCS). The cell suspension was carefully filtered through a 70µm cell strainer in a 50 mL-conical tube, and the cell strainer was washed with 30mL of perfusion buffer. Then, the cell supension was centrifuged at 400g for 20 minutes. The pelleted cells were washed and resuspended in FACS-staining buffer (PBS, supplemented with 0.5% bovine serum albumin and 2mM EDTA).

Fluorescence-activated cell sorting

To prevent capping of antibodies on the cell surface and non-specific cell labeling all steps were performed on ice and protected from light. Cells were resuspend in FACS-staining buffer and preincubated with Fc Block (Mouse BD Fc Block™; BD Biosciences) for 10 minutes. Subsequently, fluorochrome-conjugated antibodies were added and incubated for 30 minutes. The following antibodies were used: anti CD49b (clone DX5, 1:200 BD Biosciences); NK1.1 (clone PK136, 1:200 BD Biosciences); anti B220 (clone RA3-6B2, 1:200 BD Biosciences); anti CD3 (clone 145-2C11, 1:200 Biolegend); anti-CD45 (clone 104, 1:100, Biolegend/BD Biosciences); anti-CD11b (clone M1/70, 1:100 eBioscience/BD Biosciences); anti-Ly6G (clone 1A8; 1:100 Biolegend; 1:200 BD Biosciences); anti-F4/80 (clone BM8, 1:100, Biolegend; clone T45-2342, 1:100, BD Biosciences); anti–Ly-6C (clone AL-21, 1:400 BD Biosciences); anti-MHCII (clone AF6-120.1, 1:200, BD Biosciences); anti-MERTK (clone 2B10C42 , 1:100, Biolegend); anti-CD64 (clone X54-5/7.1, 1:100, BD Biosciences); anti-Ter119 (clone Ter-119, 1:100, Biolegend); anti-CD31 (clone 390, 1:100 Biolegend; 1:200 BD Biosciences); anti-NG2 (clone 1E6.4, 1:20, Miltenyi Biotec); anti-MEFSK4 (clone mEF-SK4, 1:11, Miltenyi Biotec). Finally, the cells were washed twice with ice-cold FACS-staining buffer.

After pre-selection in side scatter (SSC) vs. forward scatter (FSC) dot plot to exclude debris and FSC vs. Time-of-Flight (ToF) dot plot to discriminate doublets by gating single cells, and after gating on lineage negative ($CD49b^-/NK1.1^-/B220^-/CD3^-$) cells, macrophages were identified as $CD45^+/CD11b^+/Ly6G^-/F4/80^+$ cells, infiltrating monocytes as $CD45^+/CD11b^+/Ly6G^-/F4/80^-$cells, and neutrophils as $CD45^+/CD11b^+/F4/80^-/Ly6G^+$ cells.[1] Fibroblasts were identified as $CD31^-/TER\text{-}119^-/CD45^-/CD11b^-/NG2^-/MEFSK4^+$, endothelial cells as $CD45^-/CD11b^-/CD31^+$, vascular cells as $CD45^-/CD11b^-/CD31^-/MEFSK4^+/NG2^+$.[6] Fluorescence minus one (FMO) controls were included during acquisition for gating analyses to distinguish positive from negative staining cell populations. FACS data were acquired on a Gallios™ flow cytometer and analyzed with Gallios™ software (Beckman Coulter).

Cell sorting was performed using a FACS-Aria Fusion cell sorter (BD Biosciences), at the Research Facility Cell Sorting of Hannover Medical School. Cells were sorted in sterile

Sorting Medium (Dulbecco's modified Eagle medium supplemented with 2.5% (v/v) HI-FCS) or in Lysis-Buffer (PreEase RNA Spin Kit, Affymetrix; PN78766, USB).

Phagocytosis Assays

Neutrophils were isolated from bone marrow of C57BL/6 mice and subsequently purified by immunomagnetic selection (130-097-658, Miltenyi Biotec). To generate apoptotic neutrophils, the cells were heated at 43°C for 45 minutes and stained with Vybrant™ DyeCycle™ Violet (A35135, Invitrogen). Neutrophils obtained by this procedure were >95% apoptotic, as quantified by FACS analysis. Macrophages sorted from $MR^{flox}/MR^{LysMCre}$ infarcts were allowed to adhere to culture plates in DMEM containing 10% HI-FCS overnight. Subsequently, adherent macrophages were cultured with apoptotic neutrophils for 8 hours. After incubation the plates were washed with media to remove non-phagocytosed neutrophils and the percentage of macrophages that were Vybrant DyeCycle Violet positive was determined by flow cytometry.

Phagocytic capacity for zymosan particles of macrophages was measured using the CytoSelect™ Phagocytosis Assay (Zymosan Substrate based; CBA-224, Cell Biolabs) according to the manufacturer's instructions. Sorted infarct macrophages were allowed to adhere to culture plates in DMEM containing 10% HI-FCS overnight. Subsequently, adherent macrophages were incubated with prelabeled Zymosan particles for 2 hours at 37°C. Unbound Zymosan particles were washed and external Zymosan particles were blocked before the colorimetric detection of engulfed particles was determined using a microplate reader (Synergy-HT, BioTek).

Determination* of $O_2^{\cdot -}$ *formation

Sorted macrophages were suspended in Krebs-HEPES buffer containing 1µM hydroethidine (HE, Invitrogen) and incubated at 37°C for 30 minutes followed by centrifugation (400g, 4°C, 10 minutes). Subsequently, macrophages were washed and placed in 60µL of cold methanol containing 3,4-Dihydroxycinnamic acid (1nM) as internal standard (IS), homogenized, and then centrifuged (10000g, 4°C, 10 minutes). The superoxide-specific HE-oxidation product, 2-hydroxyethidium (2-hydroxy-E^+), was measured by ion-pair HPLC and electrochemical detection. Isocratic elution was performed (flow rate 0.8 mL/min) using a Synergi 4µ Polar-RP80A column (250x460mm, 00G-4336-E0, Phenomenex). The electrochemical detection system consisted of an ESA Coulochem III detector equipped with a Modell 5011 analytical cell (first electrode, 0.00V; second detecting electrode +0.35V). Our HPLC-EC method, incorporating an ion-pair reagent in the mobile phase, allowed more distinct separation of HE, 2-hydroxy-E^+ and ethidium (E^+). Data acquisition and analysis were performed using the Chromeleon®7 Software (Dionex). Results were normalized for cell number.[2]

Coculture of macrophages and fibroblasts

Fibroblasts sorted from infarcted myocardium of MR^{flox} and $MR^{LysMCre}$ mice, were allowed to adhere to culture plates in DMEM supplemented with 2.5% HI-FCS for 2 hours. Subsequently, using a Boyden chamber system (CLS3413-48EA, Sigma) fibroblasts and sorted macrophages from $MR^{flox}/MR^{LysMCre}$ infarcts were co-cultured in DMEM supplemented with 2.5% HI-FCS for 48 hours.

Immunocytofluorescence

For immunocytochemical staining cells were fixed for 20 minutes with BD Cytofix (5546555, BD Biosciences) and permeabilized for 7 minutes using 0.1% Triton-X in PBS supplemented with 0.5% bovine serum albumin. Antibodies included Alexa Fluor® 594-labeled anti-vimentin (7675, Cell Signaling) and FITC-labeled anti-α-SMA (F3777, Sigma). Nuclei were stained with NucBlue Live Cell Stain.

Zymography
The conditioned media was centrifuged at 4000g for 10 minutes, mixed with loading buffer and electrophoresed on SDS polyacrylamide gel containing 2mg/mL of gelatin under nonreducing conditions. Gelatinolytic bands were quantified by Image software Quantity One (Bio-Rad).[7]

Microarray
Total RNA from LV samples (infarcted or sham-operated LV myocardium) was extracted using mirVana™ miRNA isolation kit (Ambion, Applied Biosystems) following the manufacturer's protocol. RNA quality was assessed with Bioanalyzer 2100 (Agilent). RNA samples were converted to biotinylated cRNA and hybridized to GeneChip Mouse Genome 430 2.0 Array (Affymetrix) according to the manufacturer's directions. Microarray data analysis was performed using R packages from the Bioconductor project (www.bioconductor.org).[2]

RNA-Seq
Total RNA was isolated using PrepEase RNA Spin Kit (Affymetrix; PN78766, USB) according to the manufacturer's instructions with minor modifications. Sorted cells were directly collected in lysis buffer and immediately processed. RNA quantification and quality testing were assessed by NanoDrop 2000 (Thermo Fisher Scientific) and Bioanalyzer 2100 (Agilent).
Libraries for RNA sequencing were prepared from 30 ng total RNA; from each sample, poly-A RNA was purified, converted to cDNA and linked to Illumina adapters using the Illumina TruSeq stranded mRNA Kit according to the manufacturer's instructions. Samples were multiplexed and sequenced on an Illumina NextSeq 500 in a 75 nt single end setting using a high-output run mode. Raw BCL files were demultiplexed and converted to sample-specific FASTQ files using bcl2fastq v1.8.4 (Illumina). Residual adapter sequences present in the sequencing reads were removed with Cutadapt version 1.12. Reads (~ 40 million per sample) were aligned to the mouse reference sequence GENCODE vM8 using STAR version 2.5.2b. RNA sequencing data analysis was undertaken with the statistical programming language, R. The R package DeSeq2 (v1.14.1) was used to evaluate differential gene expression.[1] Candidate binding sites for mineralocorticoid receptor (MR) were identified via sequence matching using a custom R-script and Bioconductor packages: MotifDb (v1.18.0), seqLogo (v1.40.0), Biostrings (v2.44.2), GenomicFeatures (v1.28.5), org.Mm.eg.db (v3.4.1), BSgenome.Mmusculus.UCSC.mm10 (v1.4.0), TxDb.Mmusculus.UCSC.mm10.knownGene (v3.4.0). Sequences of predicted MR binding sites were extracted setting matrix score to ≥ 80% and within a region 5K bp upstream of the transcription starting site.

RT-quantitative PCR
cDNA synthesis was performed using 10 ng of total RNA and iScript™ Reverse Transcription Supermix (Bio-Rad). Relative quantitation of mRNA expression levels was determined with CFX96 Touch™ Real Time PCR using SsoAdvanced™ Universal SYBR Green Supermix and PrimePCR™ Primers (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase and beta-tubulin were chosen as an endogenous control. PCR amplification was performed at initially 95 °C for 2 min followed by 40 cycles at 95 °C for 5 s and terminated by 60 °C for 30 s. The delta-delta Ct method was employed for data analysis.[1]

Preparation of Liposomes
Liposomes were prepared as described previously by the thin-film hydration method.[8] A mixture of chloroform/methanol containing dipalmitoylphosphatidylcholine, polyethylene glycol 2000-distearoyl phosphatidylethanolamine and cholesterol was prepared in a round-

bottom flask. The lipid film was obtained evaporating organic phase by rotation under nitrogen flushing. The dry lipid film was hydrated with a solution of fluorescein isothiocyanate-labeled bovine serum albumin (liposomes encapsulating FITC-BSA), or saline (empty liposomes) or a solution of RU28318 (liposomal RU28318). Eplerenone was solubilized in chloroform/methanol and incorporated in the lipid mixture prior to lyophilization. The liposomes were extruded through a polycarbonate filter with 100 nm pore size using an Avanti Mini Extruder (Avanti Polar Lipids, Inc., USA). Unentrapped eplerenone/RU28318/FITC-BSA were removed by dialysis against PBS overnight at 4°C using Slide-A-Lyzer™ dialysis cassettes (Thermo Fisher Scientific). Phospholipid content was determined with a colorimetric assay (LabAssayTM Phospholipid, Wako).

References

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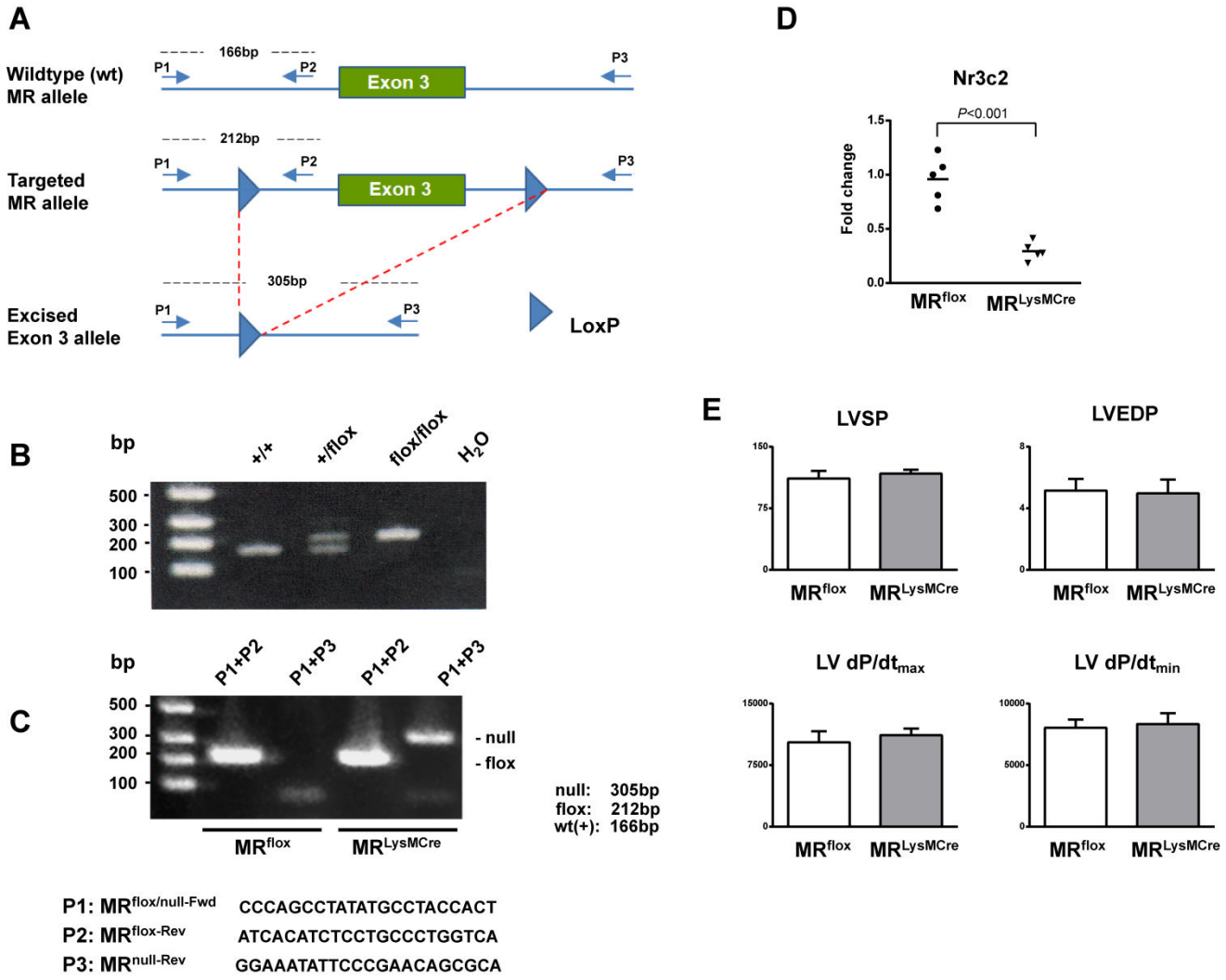


Figure S1. (**A**) Schematic representation of MR deletion strategy through Cre-Lox recombination. Floxed exon 3 region flanked by two LoxP sequences (triangles) is excised. P1, $MR^{flox/null}$ forward (Fwd) primer; P2, MR^{flox} reverse (Rev) primer; P3, MR^{null} reverse primer. (**B**) PCR showing WT and floxed MR allele. (**C**) PCR used for genotyping. (**D**) Quantitative RT-PCR of MR expression in FACS-sorted cardiac macrophages from MR^{flox} and $MR^{LysMCre}$ mice. (**E**) Left ventricular systolic pressure (LVSP), LV filling pressure (LVEDP), LV maximal rate of pressure rise (LV dP/dt_{max}), maximal rate of pressure decline (LV dP/dt_{min}), 7 days after sham operation. Mean±SEM (n=5).

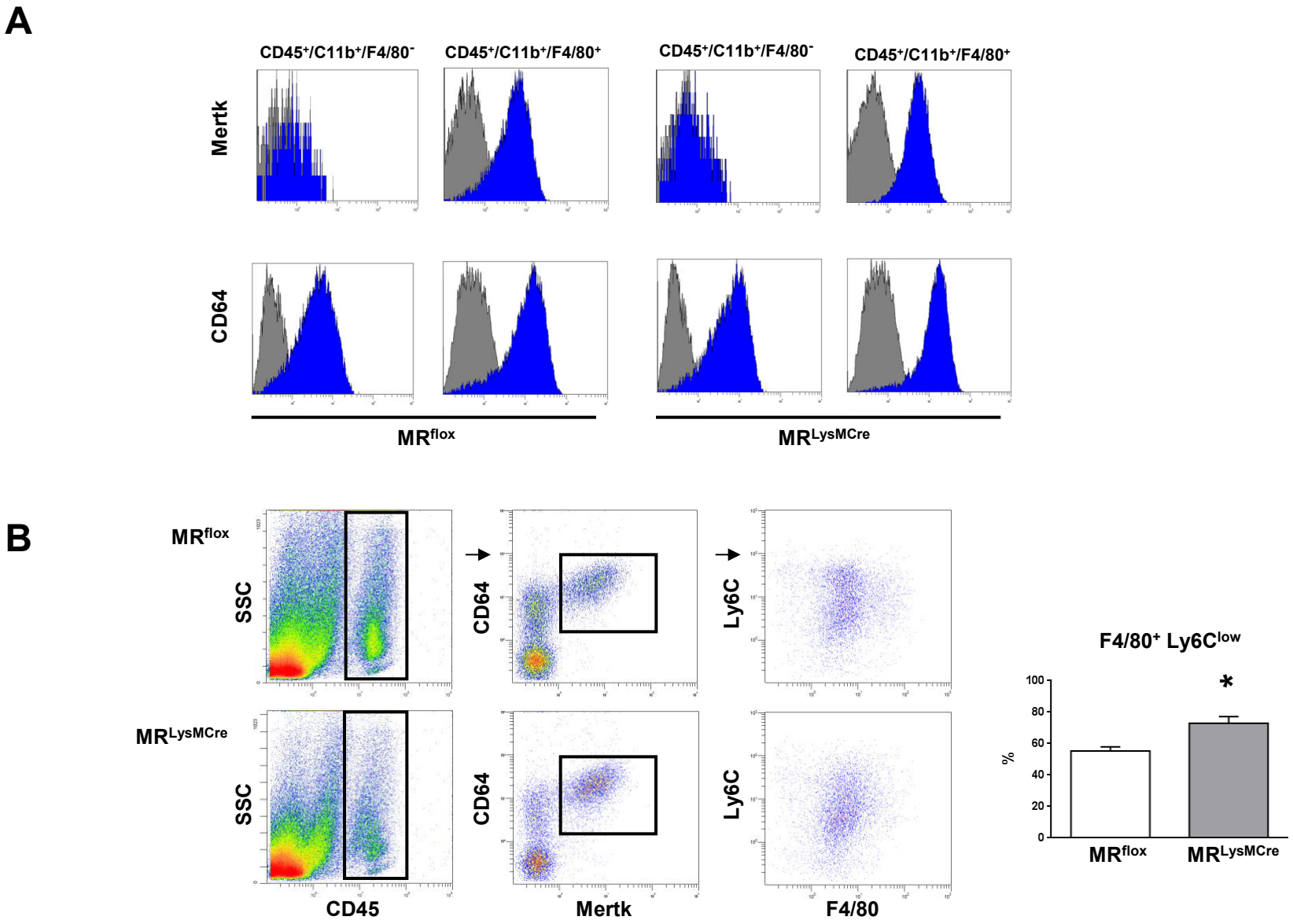
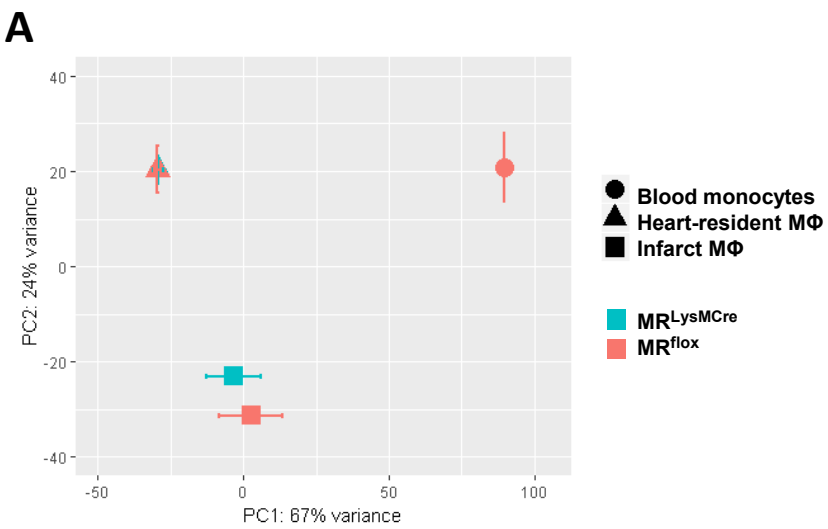


Figure S2. Characterization of monocytes/macrophages present in the infarct region of MR^{flox} and $MR^{LysMCre}$ mice, 3 days after coronary artery ligation. (**A**) $CD45^+/CD11b^+/F4/80^-$ and $CD45^+/CD11b^+/F4/80^+$ cells were analyzed for the expression of Mertk and CD64. Blue shading (specific antibody), grey shading (control). Fluorescence minus one (FMO) staining was used as controls. (**B**) Macrophages were identified as $CD45^+/Mertk^+/CD64^+$ and the proportion of $F4/80^+Ly6C^{low}$ cells was evaluated. Mean±SEM (n=3); *P<0.05 vs. MR^{flox}.



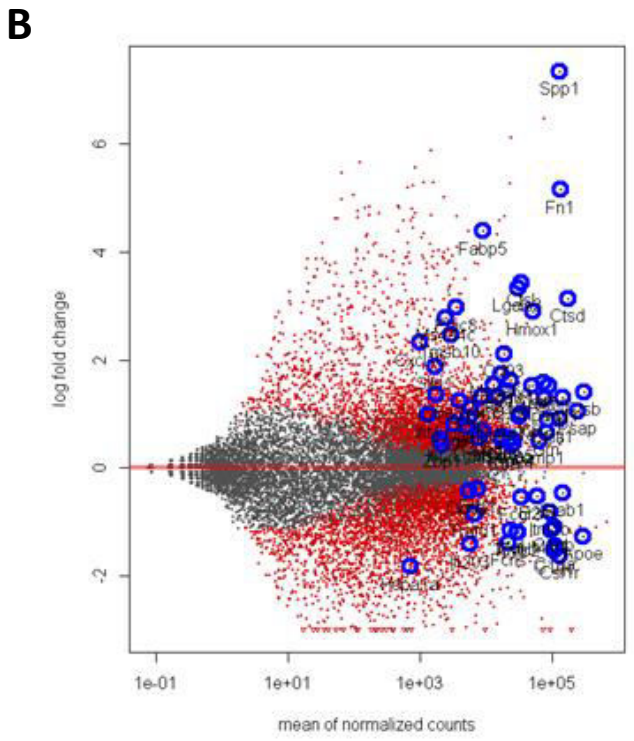


Figure S3. (**A**) Principal component analysis (PCA) comparing transcriptional profile determined by RNA-Seq of heart-resident macrophages (MΦ) at steady state (▲), infarct MΦ (■) and blood monocytes (●) from MR^{flox} and $MR^{LysMCre}$ mice. Simbols represent the centroids with standard error of the expression datasets (n=3) of each group. (**B**) MA-plot of gene expression of infarct MΦ vs. heart-resident MΦ at steady state from WT (MR^{flox}) mice. Differentially expressed genes with FDR<0.1 are depicted in red. Points circled in blue represent genes specifically expressed by macrophages present in the infarct region (from published sigle-cell RNA-seq data GSE106473: King KR, *Nat Med*. 2017;23:1481).

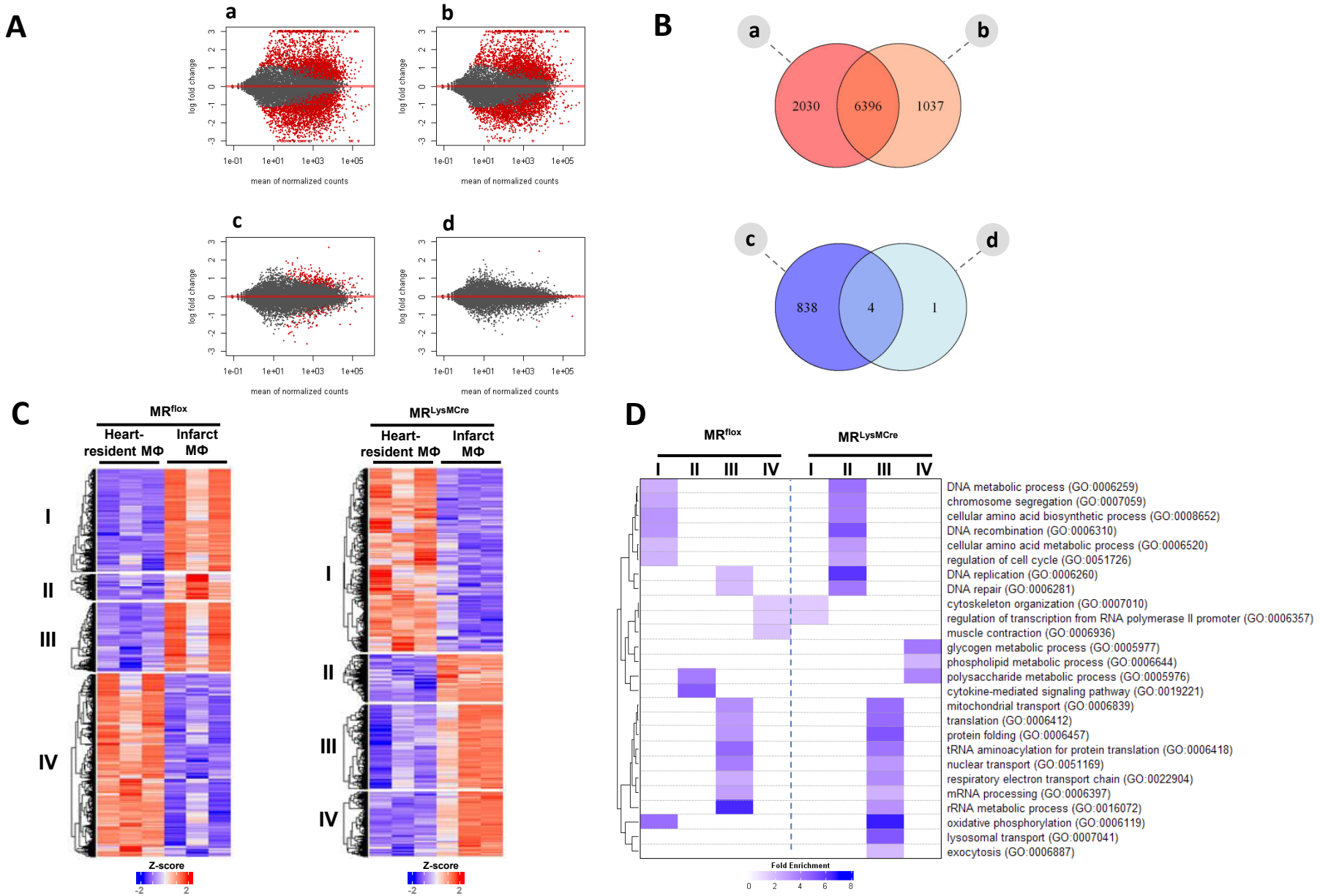


Figure S4. (**A**) MA-plot of gene expression of infarct MΦ vs. heart-resident MΦ at steady state from MR^{flox} mice (**a**) and $MR^{LysMCre}$ mice (**b**); infarct MΦ from $MR^{LysMCre}$ vs. MR^{flox} mice (**c**); heart-resident MΦ at steady state from $MR^{LysMCre}$ vs. MR^{flox} mice (**d**). Differentially expressed genes with FDR<0.1 are depicted in red. (**B**) Venn diagram of the comparisons: **a**, **b**, **c**, **d**. (**C**) Heatmap of k-means clustering of differentially expressed genes with FDR<0.1 relative to comparison (**a**, left) and comparison (**b**, right) and subsequent PANTHER pathway analysis (**D**) highlighting the most over-represented biological processes in each cluster (I to IV) with Benjamini-Hochberg padj<0.01).

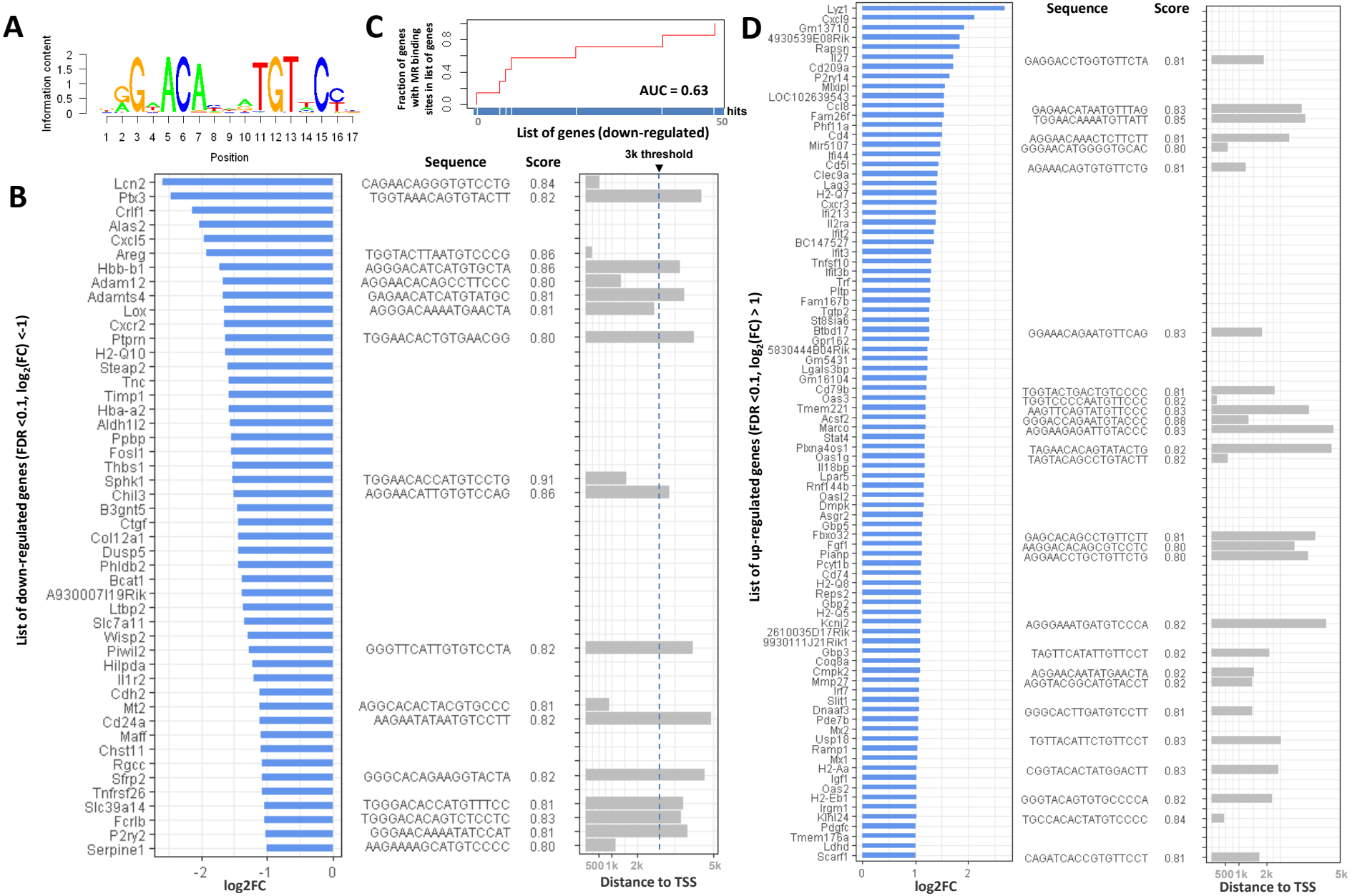


Figure S5. Candidate MR binding sites in differentially expressed genes in infarct MΦ from $MR^{LysMCre}$ vs. MR^{flox} mice with FDR<0.1 and $|\log_2(FC)| \geq 1$. (**A**) Logo of the consensus mineralocorticoid response element (MRE). (**B**, **D**) Sequences of predicted MR binding sites (≥ 80% of matrix score) along with their distances from transcription starting site (TSS). In case of gene with multiple MR binding sites the closest sequence to TSS was chosen. (**C**) ROC analysis shows a slight enrichment in down-regulated genes containing MRE motif. ROC curve was calculated taking into account only genes with potential MR binding sites with a distance to TSS less than 3K.

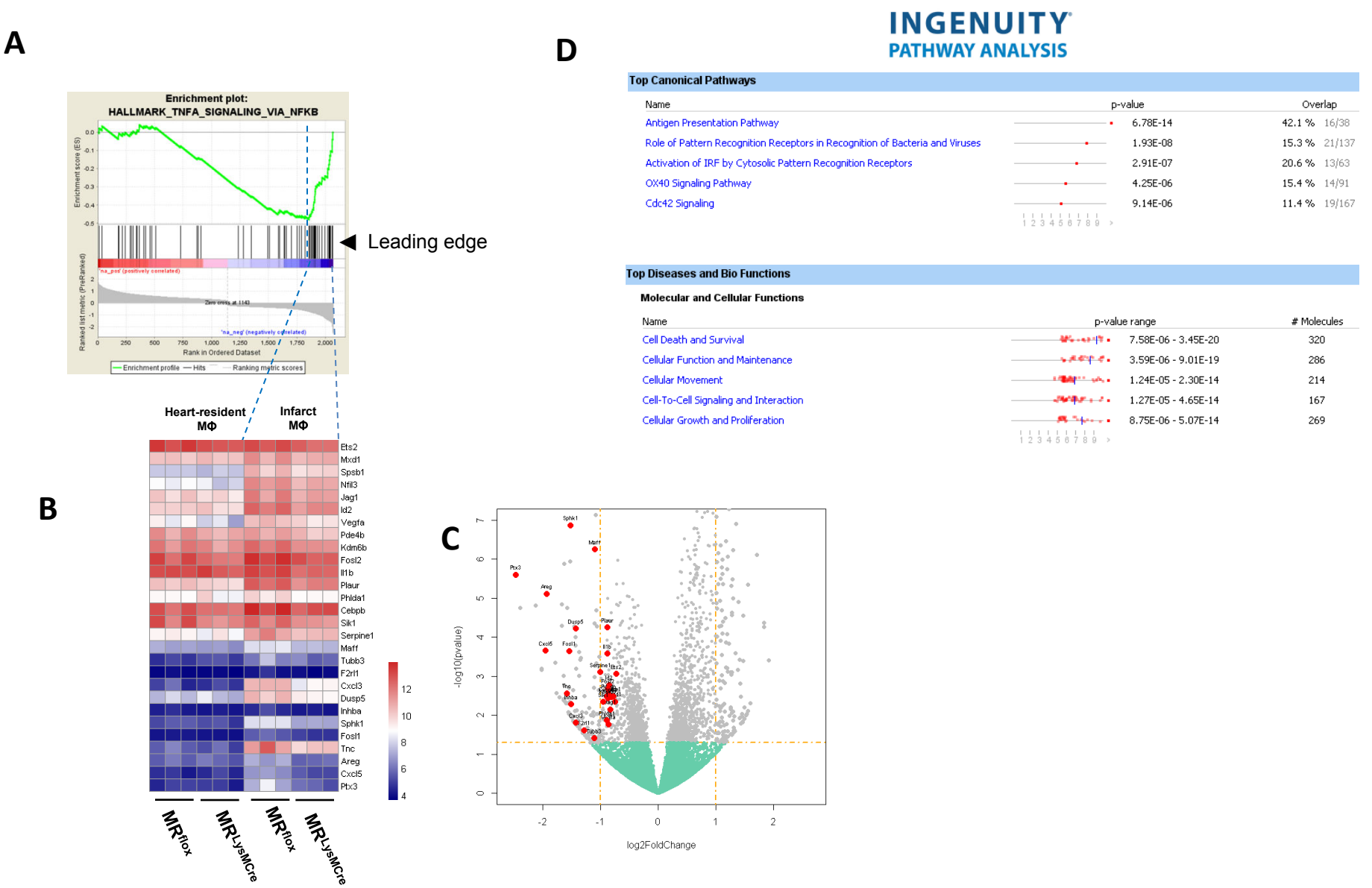


Figure S6. Pathway analyses of differentially expressed genes in infarct MΦ from $MR^{LysMCre}$ vs. MR^{flox} mice with FDR<0.1. (**A**) Gene set enrichment analysis (GSEA) revealed Tnfα signaling via NfkB among the highest significantly enriched pathways (NES = -2.81, padj<0.01). (**B**) Heatmap showing gene expression of infarct MΦ belonging to leading edge along with the relative gene expression of heart-resident MΦ at steady state. (**C**) Volcano plot of gene expression changes in infarct MΦ from $MR^{LysMCre}$ vs. MR^{flox} mice. Genes belonging to leading edge relative to Tnfα signaling via NfkB pathway are depicted in red. (**D**) Ingenuity pathway analysis reporting top five significant canonical pathways and biological functions.

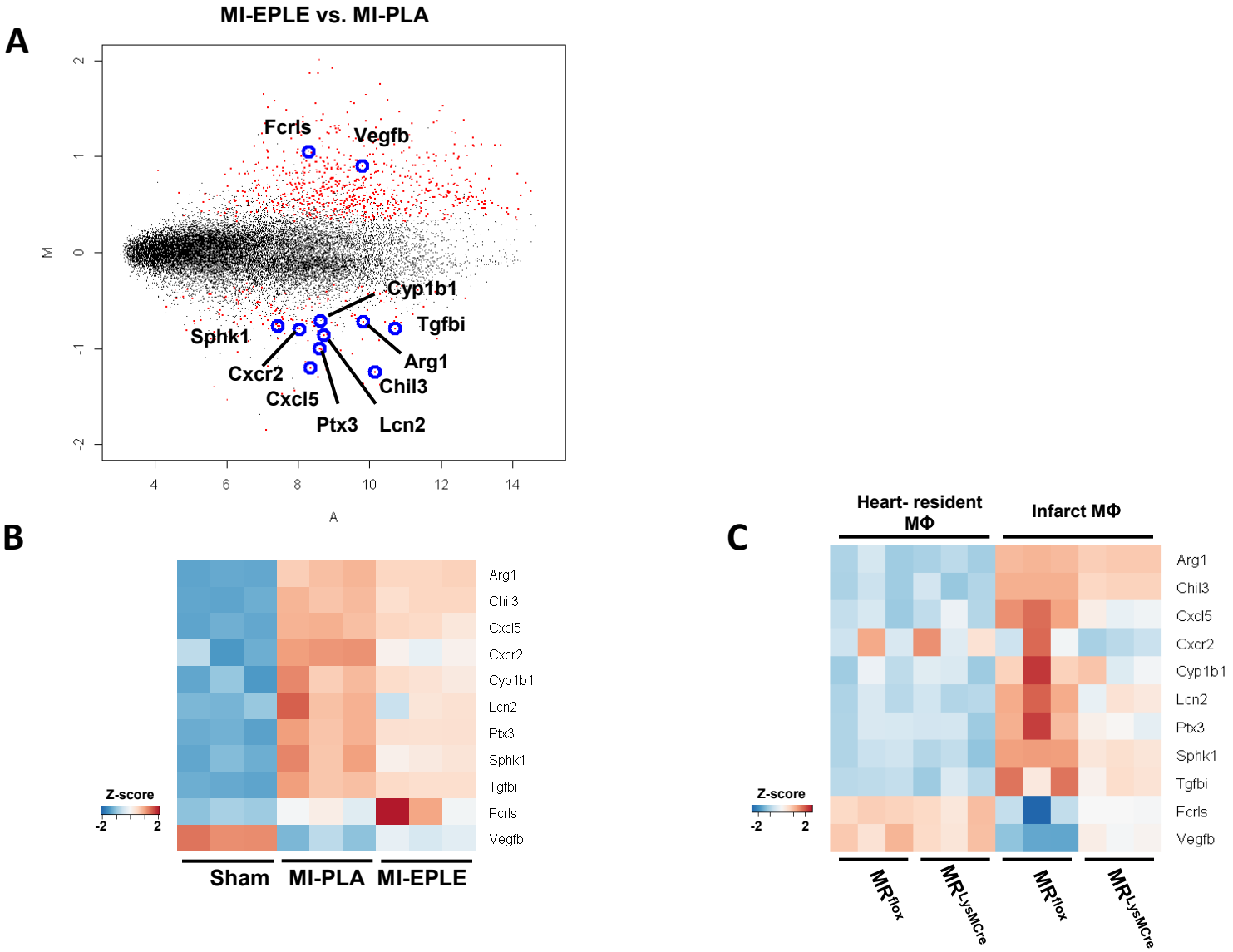


Figure S7. (A-B) Genes down/up regulated in the infarcted myocardium by eplerenone treatment 3 days after myocardial infarction (MI). Microarray analysis was performed on total RNA isolated from left ventricular myocardium of sham-operated mice (sham) and from infarcted myocardium of eplerenone (MI-EPLE) and placebo (MI-PLA) treated mice. (**A**) MA-plot of gene expression from MI-EPLE vs. MI-PLA. Differentially expressed genes with FDR<0.1 are depicted in red. (**B**) Heatmap showing expression of genes highlighted with a blue circle in the MA-plot. (**C**) Heatmap showing the expression of the same genes reported in **B** in heart-resident MΦ and in infarct MΦ from $MR^{LysMCre}$ vs. MR^{flox} mice.

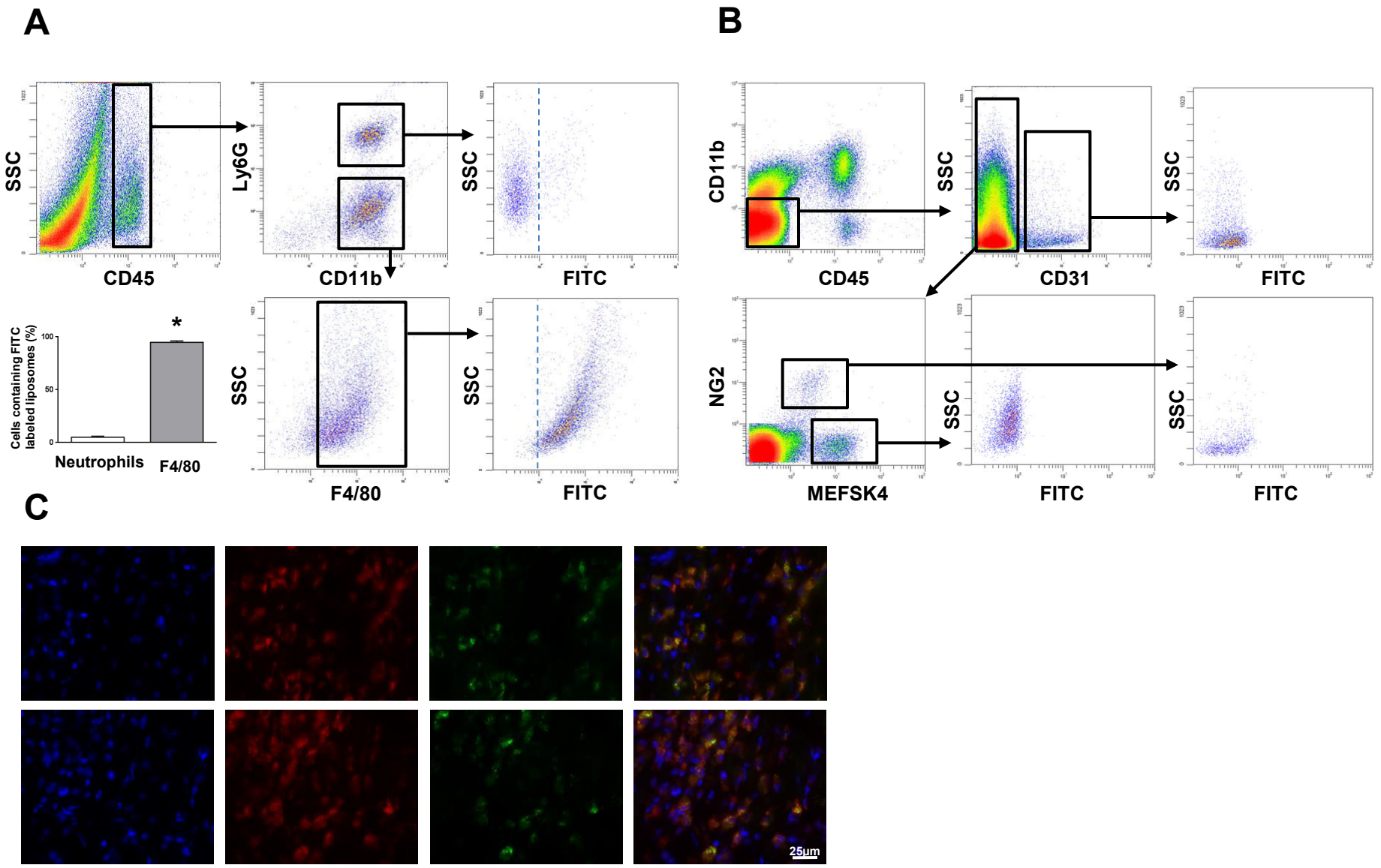


Figure S8. In vivo uptake of liposomes encapsulating FITC-BSA by macrophages in the ischemic myocardium. (**A**) Flow cytometry and gating strategy identifying infarct macrophages ($CD45^+/CD11b^+/Ly6G^-/F4/80^+$), neutrophils ($CD45^+/CD11b^+/F4/80^-/Ly6G^+$) and (**B**) endothelial cells ($CD45^-/CD11b^-/CD31^+$), fibroblasts ($CD45^-/CD11b^-/CD31^-/NG2^-/MEFSK4^+$) and vascular cells ($CD45^-/CD11b^-/CD31^-/MEFSK4^+/NG2^+$). (**C**) CD68 immunofluorescence (red), confirming that macrophages had uptaken FITC liposomes (green) in the infarcted myocardium. Nuclei were stained with NucBlue®. The liposomes were injected intraperitoneally at the onset of coronary ligation, and the *in vivo* uptake of liposomes encapsulating FITC-BSA was assessed by FACS analysis on day 1 and by immunofluorescence on day 2. Mean±SEM (n=3-4).

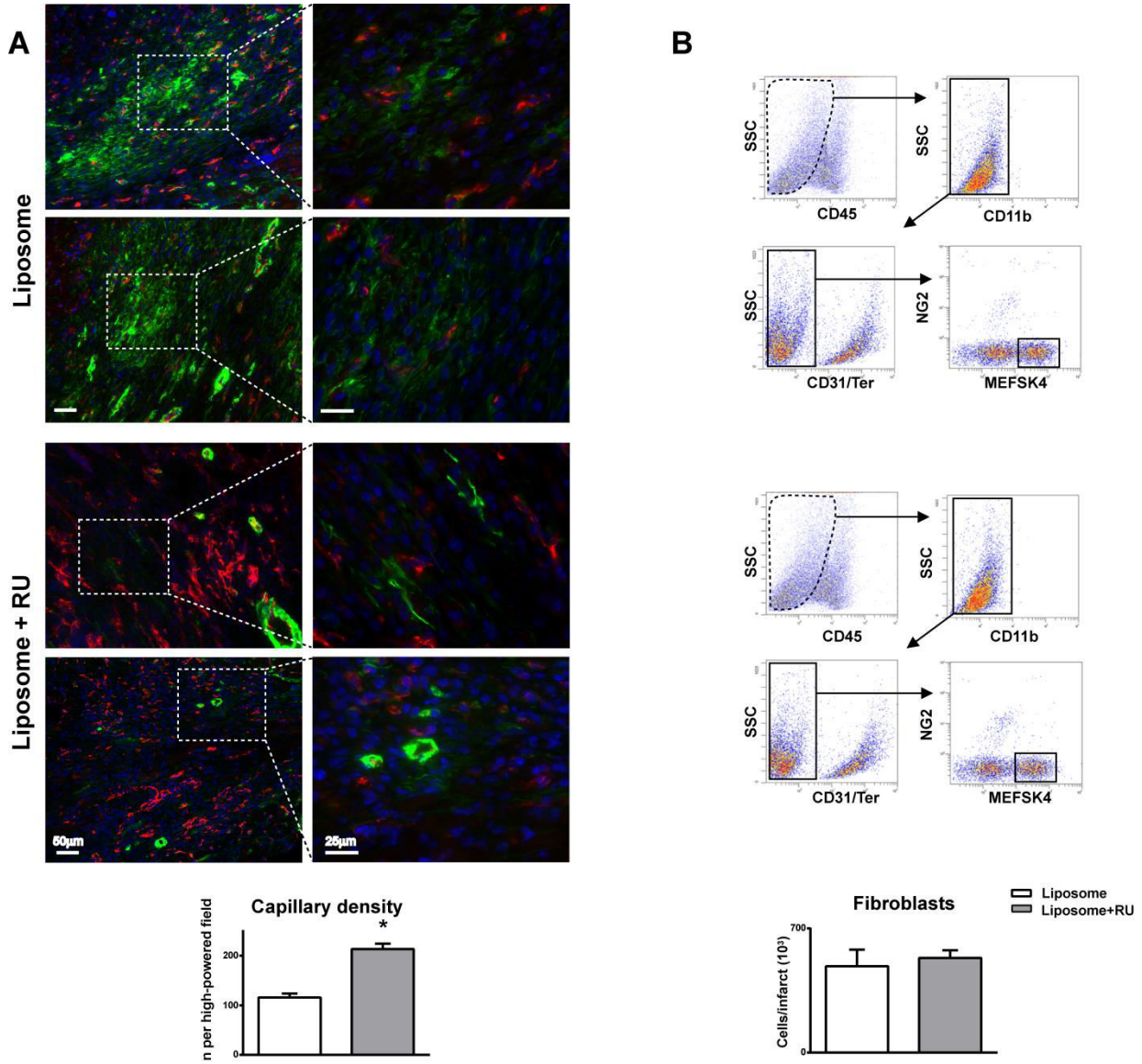


Figure S9. Targeted delivery of MR antagonists to macrophages improves the post-ischemic angiogenic response and regulates fibroblast activation state. (**A**) Immunofluorescence-double staining (CD31, red; α-SMA, green) showing capillaries, coated vessels and (myo)fibroblasts in the healing myocardium of mice receiving empty (MR antagonists-lacking) liposomes (Liposome) and liposomal RU28318 (Liposome+RU), 7 days after myocardial infarction. (**B**) FACS-sorting strategy to obtain (myo)fibroblasts from the infarct scar of mice receiving empty liposomes and liposomal RU28318. Endothelial, hematopoietic and vascular cells were excluded by selecting cells that are $CD45^-/CD11b^-/CD31^-/TER\text{-}119^-/NG2^-$. Quantitative analysis showing the number of fibroblasts present in the infarct scar. Mean±SEM (n=4-6); *P<0.05 vs. Liposome.