SUPPLEMENTAL MATERIAL

Materials and Methods

Mice

The study was approved by the local institutional animal care and use committee (IACUC). Apolipoprotein E-deficient mice ($ApoE^{-/-}$) (C57BL/6 genetic background, Jackson Laboratory) were used for this study. Male $ApoE^{-/-}$ (20-24 weeks old) C57BL/6 (12-16 weeks old) mice were used for the experiments. Mice were kept in a 22 °C room with a 12 hour light/dark cycle and received water ad libitum. To induce atherosclerosis, mice were fed a cholesterol rich, high fat diet for 5 weeks (42% calories from fat, TD. 88137). Coronary ligation was performed as described before[1,2]. After coronary ligation, mice were kept on a cholesterol rich, high fat diet for another three weeks. Mice were treated subcutaneously with a selective e-selectin inhibitor (GMI-1271, Glycomimetics) 20 mg/kg body weight twice a day for three weeks after coronary ligation. GMI-1271 is a potent, small molecule, glycomimetic antagonist for E-selectin which is now in clinical trials for hematological malignancies. Control mice were treated with PBS. After three weeks of treatment, mice were sacrificed, blood was drawn via cardiac puncture in 50 mM EDTA (Sigma- Aldrich), organs were harvested and immediately process in DPBS buffer containing 0.5% of bovine serum albumin and 1% fetal bovine serum. Red blood cells were lysed with 1x RBC lysis buffer (Biolegend). Aorta was excised under a microscope (Carl Zeiss) and minced in digestion buffer (450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I, and 60 U/ml hyaluronidase (Sigma-Aldrich)). Aortic tissue was incubated at 37°C for 1 hour and then filtered through a 40 µm nylon cell strainer (Falcon). Plasma cholesterol levels were determined with a commercially available enzymatic colorimetric assay (Cholesterol E, Wako Diagnostics). All experiments were performed in accordance with the IACUC guidelines.

Coronary Ligation

Mice were randomly assigned to either coronary ligation or the control group. Mice received preoperatively buprenorphin 0.05 mg/kg body weight. Mice were intubated and anesthetized with 2% isoflurane. Depth of anesthesia was assessed by toe pinch. The fur over the left thorax was shaved, mice were placed on a heating pad (37°C) and skin was disinfected with betadine and an alcohol wipe. The mice had been put on a ventilator before thoracotomy was carried out at the fourth left intercostal space. Pericardium was removed, and the left anterior descending coronary artery was identified and ligated with a monofilament 7-0 nylon suture to induce MI. The thorax was closed with a 5-0 suture, and the skeletal muscles as well as skin were closed with absorbable sutures. Buprenorphin twice daily was continued for three days after the procedure. The wound was monitored daily and proper recovery after surgery was ensured.

Flow cytometry

AnLSRII flow cytometer was used for flow cytometry experiments. Aortic and splenic tissues were filtered through 40 µm cell strainer and diluted in 300 µl FACS buffer to obtain a single cell suspension[1]. Staining with fluorochrome-labelled antibodies was

done in 5 ml falcon tubes (BD Bioscience). The following antibodies were used to analyze myeloid and lymphoid cells in blood: Ly6c FITC, CD115 PerCp-Cy5.5, CD11b APC-Cy7, Ly6g APC, CD3 BV421 and CD19 BV605. Ly6c positive monocytes were identified as $CD19^-$, $CD3^-$, $CD11b^+$, $CD115^+$, $Ly6g^-$ and $Ly6c^+$. Blood neutrophils were identified as $CD19^-$, $CD3^-$, $CD11b^+$, $CD115^-$, $Ly6g^+$. To determine myeloid cells in aortic tissue, two staining steps were used. First, a phycoerythrin (PE) anti-mouse lineage antibody mix containing antibodies binding CD90 (clone 53-2.1), B220, CD49b, NK1.1 and Ter-119 were used. In a second step antibodies against the following marker were applied: F4/80 PE-Cy7, CD11b APC-Cy7, Ly6g APC and Ly6c FITC. Monocytes were identified as $lineage^-$, $CD11b^+$, $Ly6g^-$, and $F4/80^-$. Neutrophils were identified as $lineage^-$, $CD11b^+$ and $Ly6g^+$. $lineage^-$, $CD11b^+$, $Ly6g^-$, and $F4/80^+$ cells in atherosclerotic plaques were considered as macrophages.

Additional antibodies were used to stain for hematopoietic stem and progenitor cells in the spleen. Besides the lineage antibodies used to stain myeloid cells, we added PE-conjugated antibodies binding CD11b, CD11c , B220, Ly-6G and IL7Rα to the lineage master mix.

In a second staining step, we used antibodies directed against c-kit, Sca-1, CD16/32, CD34, and CD115. Hematopoietic stem and progenitors cells (HSPC) were identified as $lineage^-$, $c\text{-}kit^+$ and $Sca\text{-}1^+$. HSPC that are $CD48^-$ and $CD150^+$ were considered as hematopoietic stem cells (HSC). Granulocyte and macrophage progenitors (GMPs) were identified as $lineage^-$, $c\text{-}kit^+$,$Sca\text{-}1^+$, $CD16/32^{high}$ and $CD34^{high}$. For cell cycle analysis, Ki-67 in BV605 and propidium iodide were used. Proliferating cells were identified as $Ki\text{-}67^{high}$ and propidium $iodide^{high}$. Proliferation was assessed using BrdU-flow cytometry. To this end, mice were treated with BrdU intra-peritoneally two hours before sacrifice. A commercially available BrdU kit (BD) was used to detect BrdU incorporation via flow cytometry (anti-BrdU-APC).

Histology

Aortic roots were embedded in O.C.T. medium (Sakura Finetek), snap-frozen 2-methyl butane and stored at -80°C. Aortic roots were cut with a Leica cryostat, and sections of 5 µm thickness were used for histology. CD11b staining was performed in order to determine myeloid cell accumulation in the atherosclerotic plaque. Sections were stained with anti-CD11b antibody (clone M1/70, BD Biosciences). A biotinylated anti-rat antibody served as secondary antibody (Vector Laboratories, Inc.) VECTA STAIN ABC kit (Vector Laboratories, Inc.). AEC substrate (DakoCytomation) was applied for color reaction. Accumulation of myeloid cells in atherosclerotic plaques and plaque size were quantified with iVision software. Masson Trichrome staining (Sigma Aldrich) was used to stain for necrotic cores and fibrous caps in atherosclerotic plaques. Necrotic core area was analyzed by measuring the total acellular area per atherosclerotic plaque. In order to measure fibrous cap thickness, at least 3 measurements of the thinnest fibrous cap within one atherosclerotic plaque were taken and averaged. Quantification of necrotic cores as well as fibrous cap thickness were performed using iVision software after scanning the stained sections with NanoZoomer 2.0-RS (Hamamatsu).

Magnetic resonance imaging
To ensure safety of the used substance GMI-1271 and to rule out unwanted effects on cardiac functions, we analyzed left ventricular function on day 1 and day 21 after coronary ligation using magnetic resonance imaging (MRI). We used a delayed enhancement protocol following 10-20minutes after intravenous injection of Gd-DTPA. A 7 Tesla horizontal bore Pharmascan (Bruker) that is attached to a custom-made mouse cardiac coil (Rapid Biomedical) was used to take cine images of the left ventricular short axis[2]. Analysis and quantification were done using software Segment (http://segment.heiberg.se).

E-selectin and GMI1271 interaction analysis by surface plasmon resonance
SPR measurements were performed on a Biacore X100 instrument (GE Healthcare). A CM5 sensor chip (GE Healthcare) was used for the interaction between E-selectin and GMI1271 compound. Anti-human IgG (Fc) antibody (GE Healthcare) was immobilized onto the chip by amine coupling according to the manufacturer's instructions. In brief, after a 7-min injection (flow rate of 5 µl/min) of 1:1 mixture of N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide, anti-human IgG (Fc) antibody (25 µg/ml in 10mM sodium acetate buffer, pH 5.0) was injected using a 6-min injection at 5 µl/min. Remaining activated groups were blocked by injecting 1 M ethanolamine/HCl, pH 8.5. The recombinant human E-selectin/CD63E Fc Chimera (50 µg/ml) (R & D systems) was injected into the experimental cell until 6000-7000 RU was captured onto the antibody surface. No recombinant human E-selectin/CD63E was injected into the control cell. GMI1271 samples (0-4 µM) were injected at 30 µl/min into both flow cells and all sensorgrams were recorded against the control. Regeneration of the anti- human IgG (Fc) surface was achieved by injecting 3M magnesium chloride, followed by 50 mM sodium hydroxide. Data transformation of the primary sensograms and overlay plots were prepared with BIA evaluation 4.1.1 software (GE Healthcare). Kd value was analyzed with Biacore X100 evaluation and Graphad prism software.

E-selectin binding - inhibition assay
The ability of glycomimetic compounds to inhibit binding of a polyacrylamide polymer containing Sialyl Lewis A carbohydrate to E-selectin was determined as follows. The wells of a Costar 96 well, medium binding, polystyrene assay plate (Corning 9017) were coated with 0.5 ug/mL E-selectin-Fc chimera (R&D Systems 724-ES) in Tris-buffered saline (TBS) plus 2 mM $CaCl_2$ (TBS-Ca^{2+}) for 2 hours at 37°C. The wells were blocked with TBS-Ca^{2+} plus 1% bovine serum albumin (BSA) for 2 hours at room temperature. Serial dilutions of glycomimetic compounds in TBS-Ca^{2+} were prepared in V-bottom plates (Costar 3897). Each well in the V-bottom plate containing 60 ul of compound received 60 ul of 0.3 ug/mL sialyl-Lewis A-PAA-biotin (GlycoTech 01-044) conjugated with streptavidin-horse radish peroxidase (KPL 14-30-00) (SLe^a-PAA-biotin/SA-HRP). The assay plate was washed four times with TBS-Ca^{2+} then 100 ul was transferred from the V-bottom plate to the assay plate. The plate was rocked for 2 hours at room temperature then washed four times with TBS-Ca^{2+}. Subsequently, each well received 100 ul of a two component TMB Microwell Peroxidase substrate (KPL 50-76-11). The

plates were rocked for 5 to 10 minutes at room temperature then 100 ul 1M H_3PO_4 was added to each well. The absorbance at 450 nm was measured using a FlexSation 3 (Molecular Devices). The P-selectin binding-inhibition assay was similar to the E-selectin assay, except the P-selectin-Fc chimera (R&D Systems 137-PS) was captured with anti-human IgG antibody. Briefly, wells of the assay plate were coated with 100 ul 5 ug/mL goat anti-human IgG-Fc (KPL 01-10-20) overnight at 4°C. The wells were blocked with TBS-Ca^{2+} plus 1% BSA, washed four times with TBS-Ca^{2+}, then 100 ul of 0.3 ug/mL P-selectin-Fc chimera was added to each well and allowed to bind for 1 hour at room temperature. The rest of the procedure was the same as the E-selectin assay. IC_{50} values were determined using TableCurve 2D (Systat Software, Inc.).

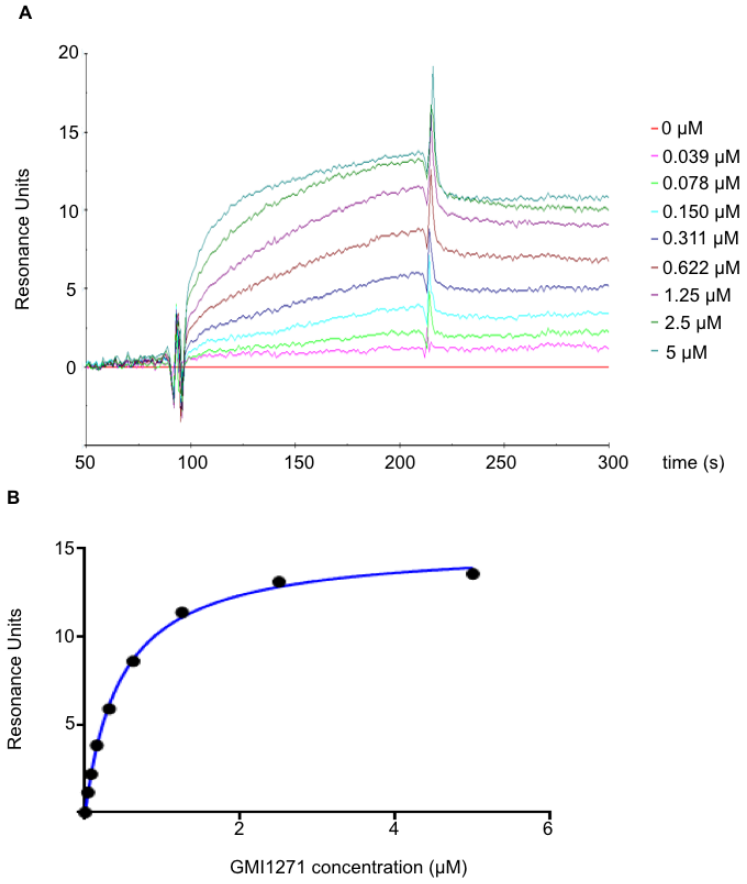
Statistics

Results are expressed as mean ± standard error of mean. The mean of the groups were compared using a non-parametric t-test (Mann-Whitney test for 2 groups) and ANOVA followed by Bonferroni test (for more than 2 groups). Differences with P values less than 0.05 were considered as statistically significant.

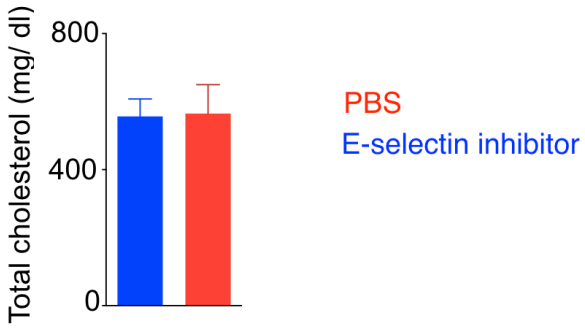
Supplemental references

1. Dutta P, Courties G, Wei Y et al. Myocardial infarction accelerates atherosclerosis. *Nature.* 2012;487:325–329.
2. Leuschner F, Rauch PJ, Ueno T et al. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *J Exp Med.* 2012;209:123–137.

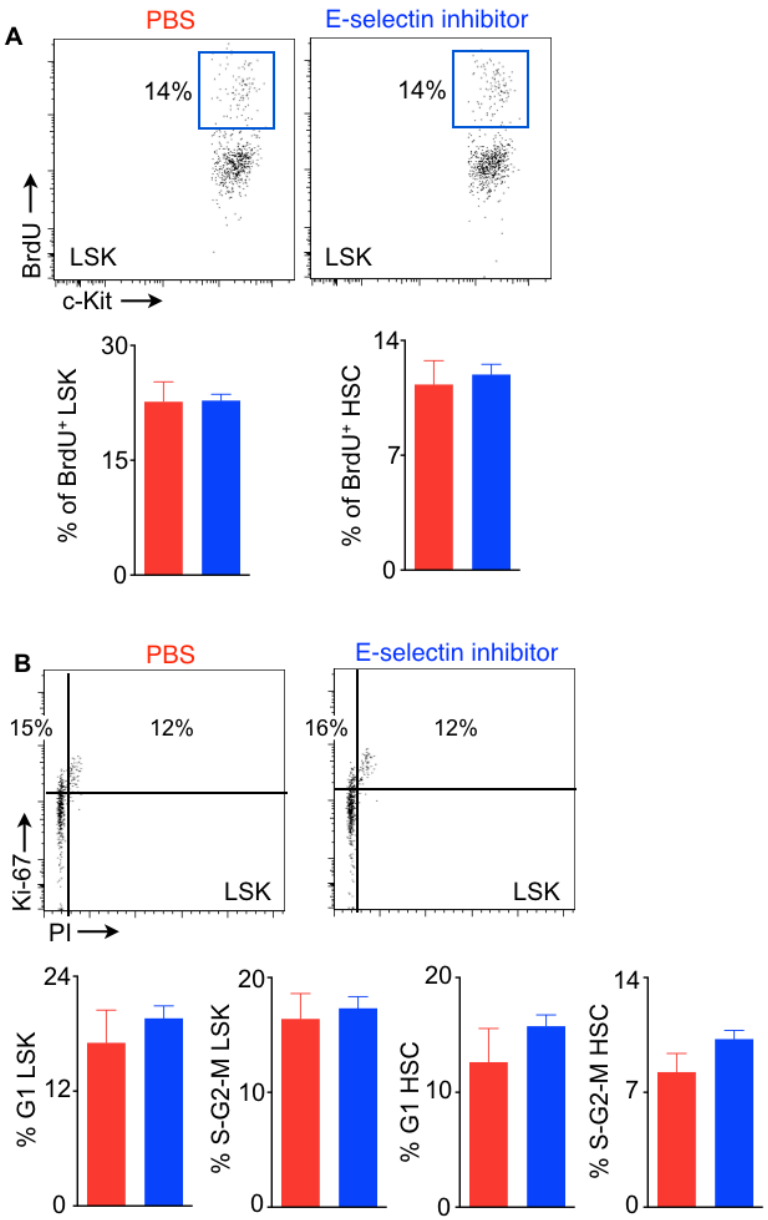
Supplemental figures



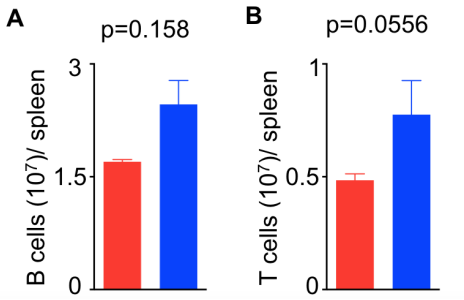
Supplemental figure I.
The dissociation constant K_D was assessed by means of surface plasmon resonance. Sensorgrams were recorded at different concentrations of GMI-1271 with a Biacore X100 instrument (A). The association, equilibrium and dissociation phases are expressed as resonance units over time. The obtained values at equilibrium were used to plot a dose-response curve (B). The concentration that induces a half-maximal response describes the dissociation constant (K_D). The K_D was determined with Biacore X100 evaluation and GraphPad prism software.



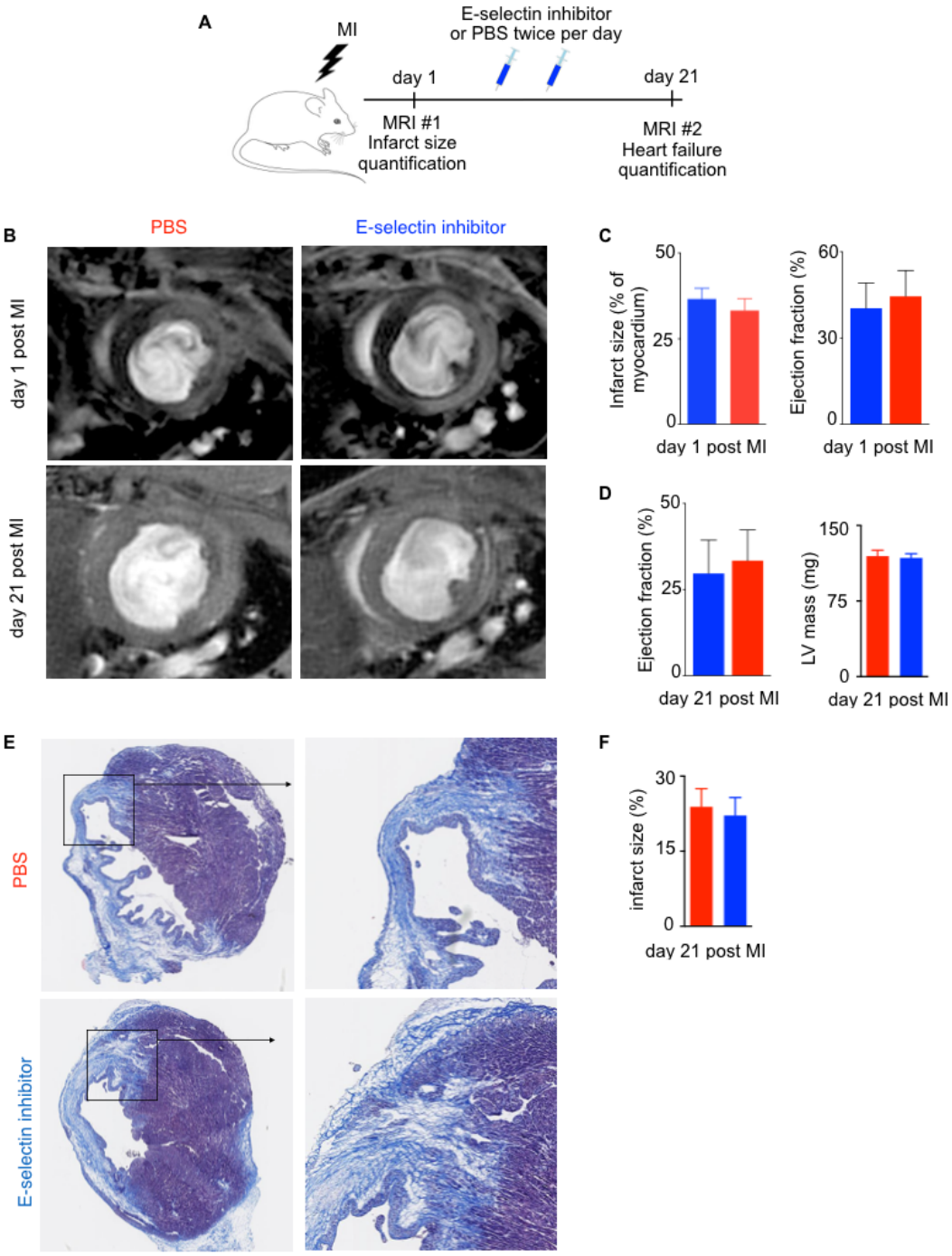
Supplemental figure II. Quantification of total blood cholesterol levels in ApoE$^{-/-}$ mice treated with either PBS or E-selectin inhibitor. Data are mean ± SEM, n=9 per group.



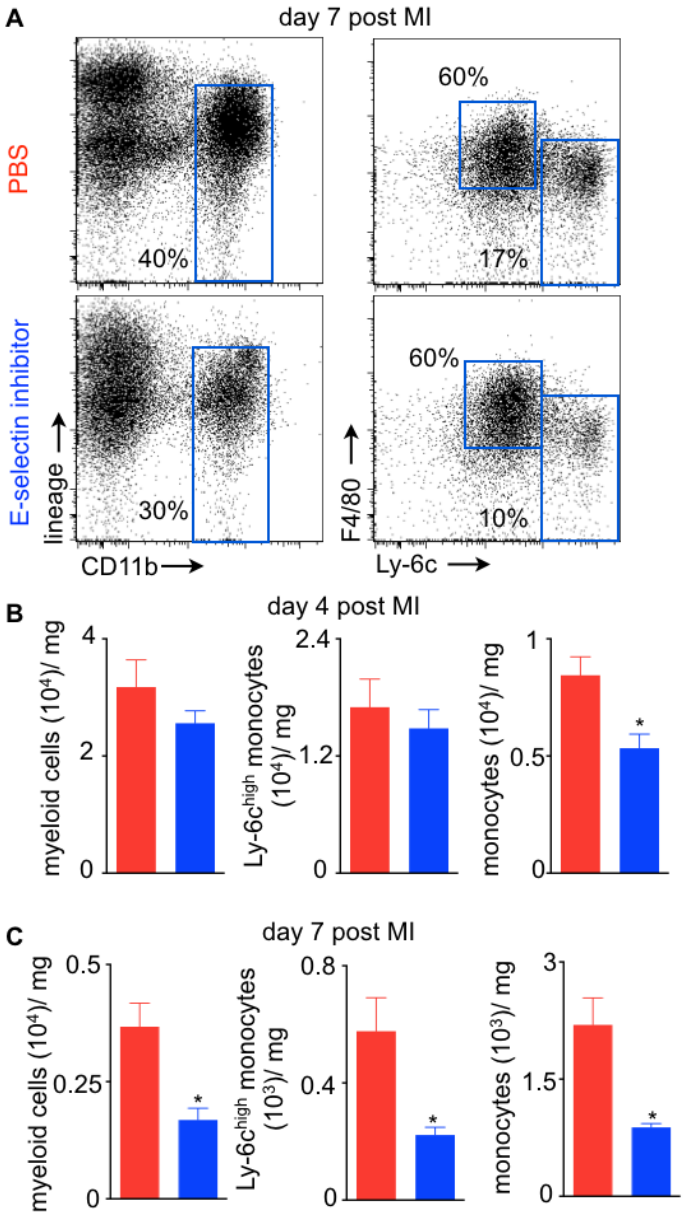
Supplemental figure III. HSC and LSK proliferative activity in the bone marrow of ApoE$^{-/-}$ mice with MI. (**A**). Proliferation of HSC and LSK assessed by BrdU-incorporation. (**B**) Cell cycle analysis by an intra-celllular staining for Ki-67 and PI. Data are mean ± SEM, n=5 per group.



Supplemental figure IV.
Lymphoid cells in the spleen were assessed by means of flow cytometry. While we observed a trend towards increased numbers of B-cells (**A**) and T-cells (**B**), the difference did not reach statistical significance. Data are mean ± SEM, n=5 per group.



Supplemental figure V. E-selectin inhibition does not affect healing of the myocardium after myocardial ischemia. (**A**) Experimental plan. (**B**) Representative MRI short axis views. (**C**) Infarct size and ejection fraction (EF) measured by MRI on day 1 after MI. (**D**) EF and left ventricular (LV) mass 21 days after MI. Data are mean ± SEM, n=16 per group.(E, F) Infarct size determined by Masson staining on day 21 after MI. Data are mean ± SEM, n=7 per group.



Supplemental figure VI.
Myocardial leukocyte accumulation was assessed by flow cytometry. (**A**) Gating strategy. (**B**) Number of myeloid cells, monocytes and Ly-6c^{high} monocytes in myocardial tissue on day 4 and (**C**) on day 7 after coronary ligation in wild type mice. Data are mean ± SEM, n=4-5 per group.