E-Selectin Inhibition Mitigates Splenic HSC Activation and Myelopoiesis in Hypercholesterolemic Mice With Myocardial Infarction

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Objective—Atherosclerosis is a chronic disease characterized by lipid accumulation in the arterial wall. After myocardial infarction (MI), atherosclerotic plaques are infiltrated by inflammatory myeloid cells that aggravate the disease and increase the risk of secondary myocardial ischemia. Splenic myelopoiesis provides a steady flow of myeloid cells to inflamed atherosclerotic lesions after MI. Therefore, targeting myeloid cell production in the spleen could ameliorate increased atherosclerotic plaque inflammation after MI.

Approach and Results—Here we show that MI increases splenic myelopoiesis by driving hematopoietic stem and progenitor cells into the cell cycle. In an atherosclerotic mouse model, E-selectin inhibition decreased hematopoietic stem and progenitor cell proliferation in the spleen after MI. This led to reduced extramedullary myelopoiesis and decreased myeloid cell accumulation in atherosclerotic lesions. Finally, we observed stable atherosclerotic plaque features, including smaller plaque size, reduced necrotic core area, and thicker fibrous cap after E-selectin inhibition.

Conclusions—Inhibiting E-selectin attenuated inflammation in atherosclerotic plaques, likely by reducing leukocyte recruitment into plaques and by mitigating hematopoietic stem and progenitor cell activation in the spleen of mice with MI. **(*Arterioscler Thromb Vasc Biol*. 2016;36:1802-1808. DOI: 10.1161/ATVBAHA.116.307519.)**

Key Words: atherosclerosis ■ E-selectin ■ HSC ■ myelopoiesis ■ myocardial infarction

Atherosclerosis is characterized by pathological lipid deposition in the arterial wall, which triggers an inflammatory cascade.[1] During progression of atherosclerosis, monocytes are recruited to lipid-rich plaques.[2] Recruitment is mediated by monocyte attachment to arterial endothelial cells that upregulate adhesion molecules, such as E-selectin, VCAM-1 (vascular cell adhesion molecule 1), and ICAM-1.[3] Leukocyte homing to atherosclerotic plaques occurs in 4 sequential steps: (1) leukocyte tethering and rolling, mediated by selectins; (2) initial leukocyte adhesion to endothelial cells via selectins and PSGL-1 (P-selectin glycoprotein ligand 1); (3) integrin-mediated firm adhesion; and (4) transmigration across the endothelial layer.[4] Selectins mediate leukocyte tethering and rolling, which are the first steps of diapedesis. Once in the plaque, inflammatory monocytes can differentiate into macrophages and produce proteases, such as collagenase, that erode the plaque's fibrous cap. Such erosions leave plaques vulnerable to rupture that results in myocardial infarction (MI).

Recurrent ischemic injuries occur frequently and are often fatal.[5] In ApoE$^{-/-}$ mice, coronary ligation leads to extramedullary myelopoiesis that persists even 12 weeks after the ischemia.[6] Inflammatory myeloid cells produced in the spleen are recruited to atherosclerotic plaques via blood.[7] In ApoE$^{-/-}$ mice, MI increases plaque size, plaque monocyte numbers, and alters monocyte phenotype as monocytes isolated from atherosclerotic lesions after MI express higher levels of inflammatory genes.[6] Subsequent studies, using angiography to measure the progression of nonculprit coronary artery lesions in patients with ST-segment–elevation MI,[8] showed that these patients had faster advancing lesions when compared with a cohort without MI.

Because myeloid cells have high turnover rates in inflammatory tissues,[7,9] they must be replenished by hematopoietic stem and progenitor cells (HSPC). MI diminishes bone marrow levels of hematopoietic stem cell (HSC) retention factors, such as CXCL12 (chemokine C-X-C motif ligand 12) and VCAM-1, triggering HSPC release from the bone marrow. The released HSPC seed the spleen and divide in the presence of stem cell factor to produce myeloid cells that, after being recruited to atherosclerotic plaques, render the lesions more inflamed.[6] This understanding of HSPC's role after MI is supported by clinical data showing higher splenic uptake of the PET (positron

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Nonstandard Abbreviations and Acronyms	
HSC	hematopoietic stem cells
HSPC	hematopoietic stem and progenitor cells
MI	myocardial infarction

emission tomography) tracer ^{18}F-FDG (fluorodeoxyglucose), which is incorporated by cells with higher energy demands, such as proliferating cells. The spleens and bone marrow of patients with acute MI showed significantly higher PET tracer uptake.[10] Although ^{18}F-FDG is not specific to a certain cell type, these results may indicate increased cell proliferation in the spleen in patients with acute MI. In a retrospective analysis, patients with higher splenic ^{18}F-FDG uptake showed increased cardiovascular events after imaging.[11] These studies indicate that splenic activation plays a role in increased atherosclerotic lesion inflammation after MI. However, there is currently no clinically available intervention that can block this process.

HSC proliferation and differentiation are tightly regulated by niche cells, such as endothelial cells, mesenchymal stem cells, among others, in the bone marrow microenvironment.[12] E-selectin, a cell adhesion molecule expressed by sinusoidal bone marrow endothelial cells, regulates HSC fate by promoting progenitor proliferation.[13] HSC proliferation decreased in E-selectin knockout mice and after pharmacological E-selectin inhibition. Whether similar mechanisms are active in the spleen is not well understood. We hypothesized that E-selectin inhibition dampens splenic monocyte production in hypercholesterolemic mice with experimental MI. Previously, we found that MI transiently elevated splenic progenitor proliferation after MI in wild-type mice,[9] and more permanently in ApoE$^{-/-}$ mice after coronary ligation,[6] both resulting in increased extramedullary myelopoiesis. In the current study, we describe that inhibiting E-selectin with a small-molecule glycomimetic antagonist (GMI-1271) significantly decreased HSC proliferation in the spleens of mice with atherosclerosis and MI. Reduced proliferation led to lower HSC and progenitor numbers and attenuated splenic myeloid cell output. Furthermore, after E-selectin inhibition, aortic plaque contained significantly fewer inflammatory myeloid cells. The treatment reduced atherosclerotic plaque size and necrotic core area, whereas plaque fibrous caps were thicker.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

MI Triggers Splenic HSPC Proliferation

To investigate the time course of upstream splenic HSPC proliferation after MI, we ligated the left anterior descending coronary artery. At different time points after MI, we injected wild-type mice with BrdU (bromodeoxyuridine), a thymidine analogue that can be incorporated into DNA strands during the S phase of cell cycle. We found that splenic HSPC proliferation progressively increased after coronary ligation. It peaked on day 3 and returned to steady-state level on day 7 (Figure 1A–1C). On day 2 after coronary ligation, HSPC proliferation was >4× higher than in mice without coronary ligation (control, 4.06±0.4 BrdU$^+$ HSPC; day 3 after coronary ligation, 17.3±1.9%). Concomitantly, splenic myeloid cell numbers increased at day 4 after myocardial ischemia (Figure 1D), thereby indicating that proliferating HSPC differentiated into myeloid cells.

E-Selectin Inhibition Decreases Splenic HSC and Progenitor Proliferation

Splenic HSC and progenitor proliferation fuels extramedullary myelopoiesis.[7,14] However, the regulation of splenic HSC proliferation (Figure 2A) is not well understood. Because E-selectin expressed by bone marrow sinusoidal endothelial cells promotes HSC proliferation,[13] we hypothesized that inhibiting E-selectin would reduce splenic HSC proliferation. To test this hypothesis, we treated C57BL/6 mice after MI with a small-molecule E-selectin inhibitor (GMI-1271). Surface plasmon resonance revealed that GMI-1271 potently inhibited E-selectin with a K_D in the high nanomolar region (0.46 µmol/L). GM-1271's IC50 for E-selectin was 1.75 µmol/L, whereas it was 2.9 mmol/L for L-selectin and >10 mmol/L for P-selectin. These data indicate specificity of GMI-1271 for E-selectin (Figure I in the online-only Data Supplement). Treatment with GMI-1271 after coronary artery ligation resulted in significantly decreased splenic proliferation of HSC (PBS, 26.4±1.7%; GMI-1271, 17.6±3.2%) and HSPC (PBS, 26.5±1.1%; GMI-1271, 21.3±3.9%; Figure 2B).

We recently described that MI triggers splenic HSC proliferation,[9] leading to increased inflammatory myeloid cell production that exacerbates atherosclerosis.[6] To investigate the role of E-selectin in splenic HSC proliferation in atherosclerotic mice, we next performed coronary ligation in ApoE$^{-/-}$ mice fed a high-fat diet. After MI, the mice received E-selectin inhibitor injections. GMI-1271 treatment did not alter blood cholesterol levels (Figure II in the online-only Data Supplement). Cell cycle analysis was done on day 21 after MI (Figure 2C). HSPC and HSC were stained for Ki-67, an antigen expressed in cycling cells. DNA marking with PI distinguished cells in S-G2-M phase (ie, proliferating cells) from those in G1 phase (Figure 2D). We found that E-selectin inhibition decreased the percentage of HSPC in G1, S-G2-M, and non-G0 phases, whereas the percentage of HSPC in G0 stage was increased (Figure 2E). We detected similar effects on splenic HSC proliferation (Figure 2F). Interestingly, GMI-1271 treatment did not change bone marrow HSC and HSPC proliferation (Figure III in the online-only Data Supplement) in the same cohort of ApoE$^{-/-}$ mice with MI.

E-Selectin Inhibition Lowers Splenic HSC and Progenitor Numbers

Because inhibiting E-selectin decreased HSC and HSPC proliferation, we investigated whether the treatment also decreased their numbers. For this experiment, we again used ApoE$^{-/-}$ mice fed a high-fat diet. Figure 3A depicts the gating strategy for HSPC (Lin$^-$ c-Kit$^+$ Sca-1$^+$), HSC (Lin$^-$ c-Kit$^+$ Sca-1$^+$ CD48$^-$ CD150$^+$), and granulocyte–monocyte progenitors (Lin$^-$ c-Kit$^+$ Sca-1$^-$ CD16/32$^+$ CD34$^+$). GMI-1271 treatment significantly reduced splenic numbers of HSPC (PBS, $47\times10^3\pm15\times10^3$; GMI-1271, $20\times10^3\pm2\times10^3$), HSC (PBS, $4\times10^3\pm1\times10^3$; GMI-1271,

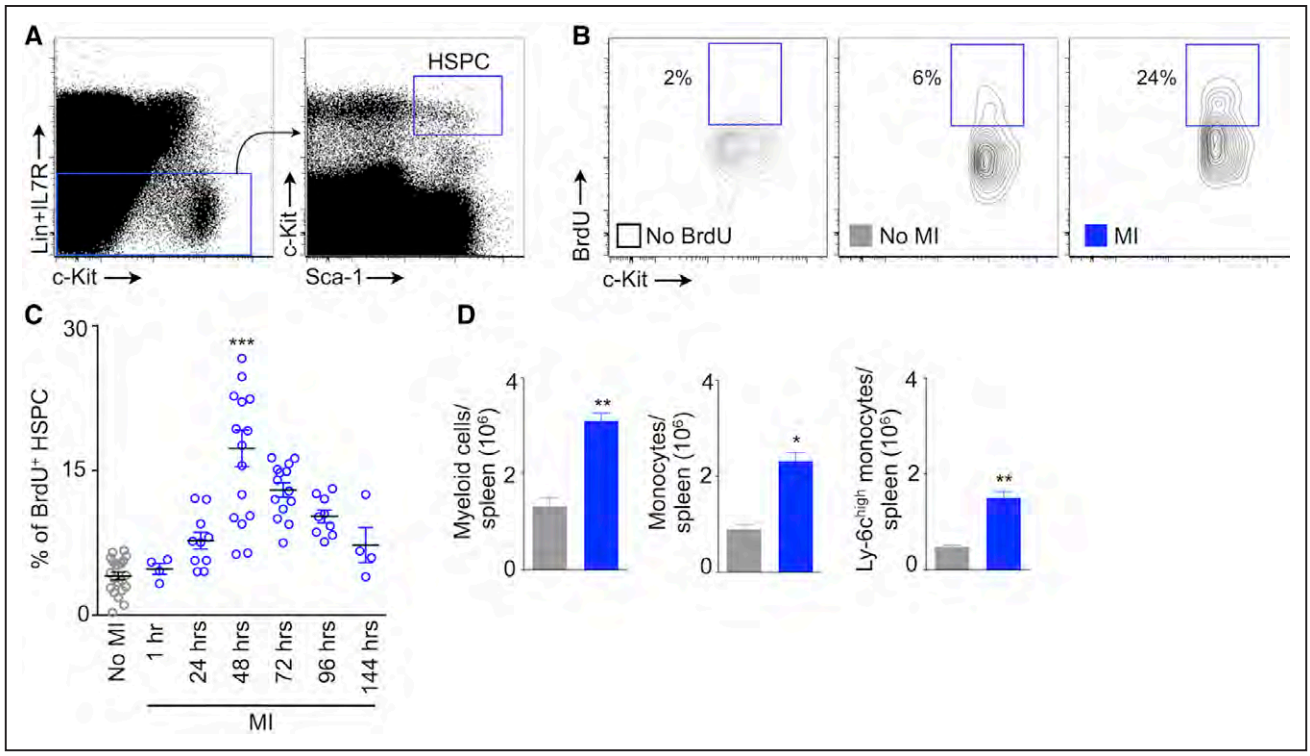

Figure 1. Myocardial infarction (MI) triggers splenic hematopoietic stem and progenitor cell (HSPC) proliferation. **A**, Flow cytometry gating strategy for splenic HSPCs. **B**, Representative flow cytometric plots showing BrdU+ (bromodeoxyuridine) splenic HSPC. BrdU was injected 48 hours after MI. **C**, The graph depicts % of BrdU+ splenic HSPC. BrdU was injected at different time points as indicated on the *x* axis. Each data point indicates one mouse, ***P<0.001. **D**, Quantified expansion of splenic myeloid cells 4 days after MI. n=4 to 8 per group. Data are mean±SEM, *P<0.05, **P<0.01.

$2\times10^3\pm0.2\times10^3$), and granulocyte–monocyte progenitors (PBS, $23\times10^3\pm6\times10^3$; GMI-1271, $8\times10^3\pm1\times10^3$; Figure 3B).

E-Selectin Inhibition Dampens Myelopoiesis

MI-induced activation of splenic HSPC contributes to a systemic rise in myeloid cell numbers,[6] and the spleen supplies myeloid cells in the setting of atherosclerosis.[7,15] Because E-selectin inhibition significantly reduced HSC and HSPC proliferation in the spleen, we hypothesized that the treatment would also reduce circulating myeloid cell numbers in mice with MI. Indeed, GMI-1271 treatment significantly reduced myeloid cell numbers in the blood (Figure 4A and 4B). Figure 4A shows the gating strategy for monocytes, the inflammatory Ly-6c^{high} monocyte subset, and neutrophils. In Apoe$^{-/-}$ mice, E-selectin inhibition reduced total monocytes, Ly-6c^{high} monocytes, and neutrophil levels by 28%, 41%, and 40%, respectively, as measured 3 weeks after MI (Figure 4B). Interestingly, ApoE$^{-/-}$ mice treated with GMI-1271 showed a trend toward increased lymphocyte numbers (Figure IV in the online-only Data Supplement), which may indicate that E-selectin inhibition influences hematopoietic lineage bias, proliferation of lymphocytes, or lymphocyte migration.[16]

Mitigated Inflammation in Atherosclerotic Lesions and Enhanced Stable Plaque Phenotype After E-Selectin Inhibition

Atherosclerosis is associated with systemically increased inflammatory myeloid cells[15,17–19] that are recruited to atherosclerotic plaques, a process that partially depends on E-selectin.[4,20,21] The presence of inflammatory cells is detrimental to a stable plaque.[22] Because the E-selectin inhibitor effectively curbed myelopoiesis (Figure 4), we next investigated whether GMI-1271 treatment might reduce inflammation in atherosclerotic lesions, thus, promoting stable plaques. To test this hypothesis, we induced MI in ApoE$^{-/-}$ mice fed a high-fat diet. ApoE$^{-/-}$ mice were injected with either PBS or GMI-1271 for 3 weeks after coronary ligation. E-selectin inhibition significantly reduced aortic accumulation of myeloid cells, Ly-6c^{high} monocytes, macrophages, and neutrophils (Figure 5A and 5B). Diminished lesion inflammation was confirmed by histology, which revealed a smaller CD11b-stained area in sections of the aortic root (Figure 6A and 6B). Furthermore, plaque size in the aortic root decreased significantly (Figure 6C).

Matrix metalloproteinases produced by inflammatory myeloid cells erode the fibrous cap, thereby allowing blood to come into contact with thrombogenic materials in the underlying necrotic core.[23] This may result in acute coronary thrombosis and myocardial ischemia. Accordingly, E-selectin inhibitor treatment might promote stable plaque features, such as small necrotic cores and thick fibrous caps. To test this hypothesis, we performed Masson staining of aortic root sections from ApoE$^{-/-}$ mice 3 weeks after MI. We found that GMI-1271 treatment significantly reduced necrotic core area and increased fibrous cap thickness (Figure 6D and 6E).

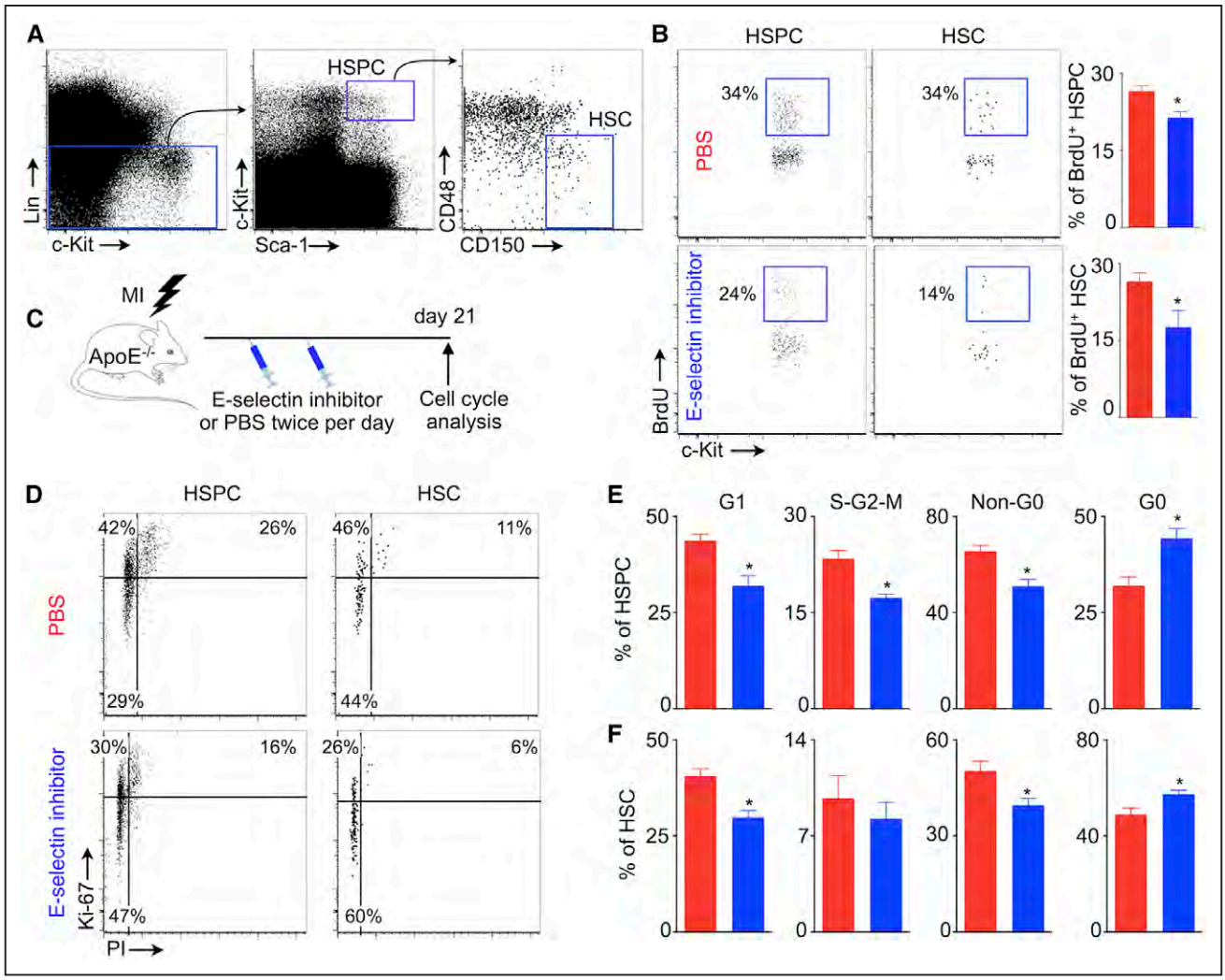

Figure 2. E-selectin inhibition decreases splenic hematopoietic stem cell (HSC) and progenitor proliferation. **A**, HSC gating strategy. **B**, Percent of BrdU+ (bromodeoxyuridine) hematopoietic stem and progenitor cells (HSPC) and HSC in the spleens of C57BL/6 mice. BrdU was injected 48 hours after coronary ligation. **C**, Experimental design to investigate the effects of E-selectin inhibitor on HSPC and HSC proliferation in atherosclerotic mice with myocardial infarction (MI). **D**, Representative flow cytometric plots depicting HSPC and HSC proliferation after MI in ApoE$^{-/-}$ mice treated with either PBS or E-selectin inhibitor. **E**, Percent of splenic HSPC and HSC in different phases of the cell cycle. Data are mean±SEM, n=5 per group, *P<0.05.

We also investigated the impact of E-selectin inhibition on myocardial healing after infarction by inducing MI in C57BL/6 wild-type mice. Cardiac magnetic resonance imaging was used to quantify infarct size on day 1 and measure heart failure on day 21 after MI (Figure VA–VD in the online-only Data Supplement). Initial infarct size was determined on day 1 after coronary artery ligation by delayed gadolinium enhancement and was similar for both groups (Figure VC in the online-only Data Supplement). That mice in the GMI-1271- and PBS-treated groups had similar ejection fraction (Figure IVD in the online-only Data Supplement) indicates drug safety for the post-MI phase. Masson histology on day 21 after coronary ligation confirmed similar infarct size in both treatment groups (Figure VE in the online-only Data Supplement). The treatment with GMI-1271 reduced infarct leukocytes, especially on day 7 after coronary ligation (Figure VI in the online-only Data Supplement).

Discussion

The adhesion molecule E-selectin is known for its role in recruiting inflammatory leukocytes into the arterial wall. In the setting of atherosclerosis, E-selectin is expressed by activated endothelial cells and binds carbohydrate ligands on leukocytes, leading to cell adhesion to the endothelial surface. Interleukin-1 and tumor necrosis factor-α are known instigators of E-selectin expression by endothelial cells.[24] The adhesion molecule promotes a critical step in monocyte recruitment and, thus, contributes to plaque inflammation.[4] Recently, it was shown that E-selectin also promotes HSC activity in the bone marrow and that its inhibition can reduce hematopoietic progenitor activity in this organ.[13] Here we describe that E-selectin also regulates splenic myelopoiesis in mice with atherosclerosis and MI.

The spleen serves as a source of monocytes in ischemic heart disease.[25] Hyperlipidemia[7] and myocardial injury[9] lead to hematopoietic progenitor retention in this organ, which

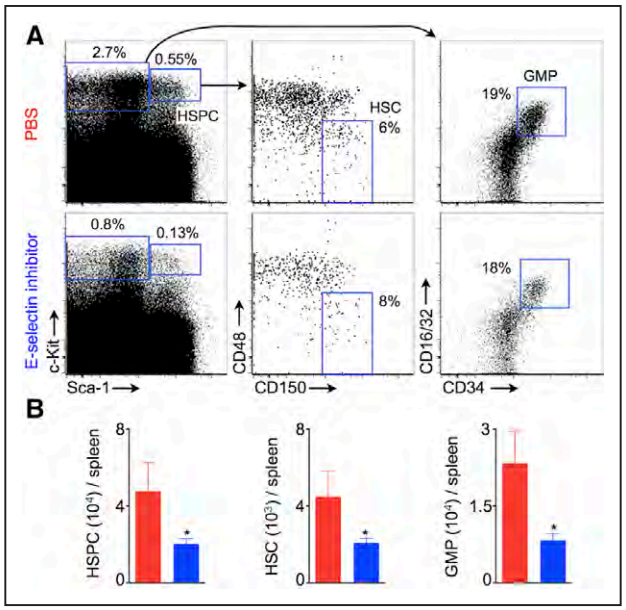

Figure 3. E-selectin inhibition decreases splenic hematopoietic stem cell (HSC) and progenitor numbers. **A**, Flow cytometric gating strategy for splenic HSC and granulocyte–monocyte progenitors (GMP). **B**, Quantification of hematopoietic stem and progenitor cells (HSPC), HSC, and GMP in the spleens of ApoE$^{-/-}$ mice 3 weeks after coronary ligation. The mice were treated with either PBS or E-selectin inhibitor twice a day for 3 weeks. Data are mean±SEM, n=5 per group, *P<0.05.

consequently supplies myeloid cells to the growing atherosclerotic plaque and to ischemic myocardium. Although first clinical data indicate splenic activation in patients with acute coronary syndrome,[11] the regulation of splenic hematopoiesis is incompletely understood. Given previous reports

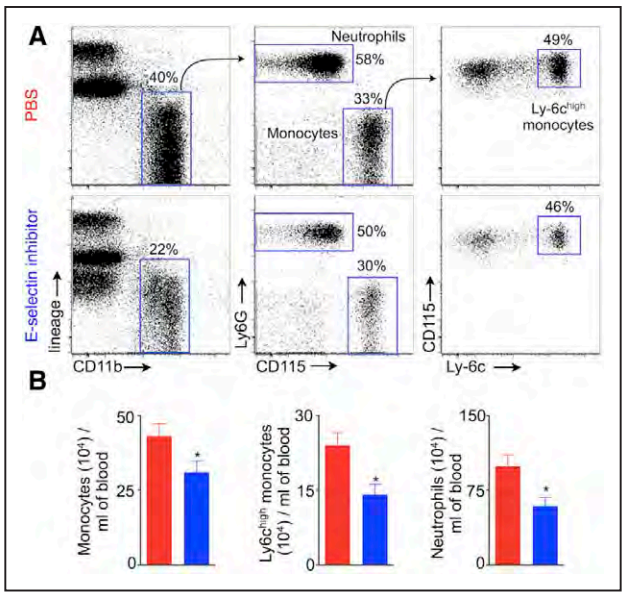

Figure 4. E-selectin inhibition reduces blood leukocytosis in mice with atherosclerosis and myocardial infarction (MI). **A**, Flow cytometric gating strategy for neutrophils, monocytes, and Ly-6c^{high} monocytes. The flow cytometric plots depict % of myeloid cells in the blood of ApoE$^{-/-}$ mice treated with either PBS or E-selectin inhibitor. **B**, Enumeration of neutrophils, monocytes, and Ly-6c^{high} monocytes in ApoE$^{-/-}$ mice 3 weeks after MI. Data are mean±SEM, n=5 per group, *P<0.05.

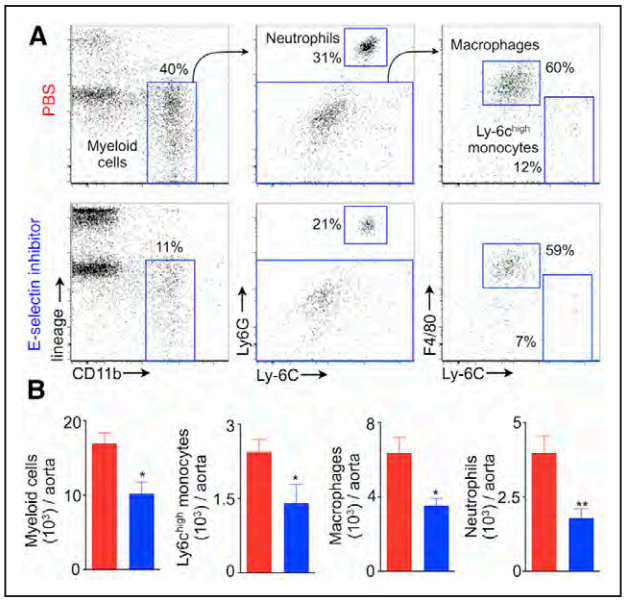

Figure 5. E-selectin inhibition mitigates inflammation in atherosclerotic plaques. Aortas were excised from ApoE$^{-/-}$ mice fed a high-fat diet 3 weeks after coronary ligation. **A**, The flow cytometric plots depict gating strategy and % of different myeloid cells in the aorta. **B**, Quantification of aortic myeloid cells, Ly-6c^{high} monocytes, macrophages, and neutrophils. Data are mean±SEM, n=9 per group, *P<0.05, **P<0.01.

on E-selectin upregulation after MI[26] and its association with hyperlipidemia and cardiovascular disease in general,[27] we studied the effects of the E-selectin inhibitor GMI-1271, which has a good safety profile in humans,[28] in mice with MI and preexisting atherosclerosis. Previous work established that in this scenario, which mimics the occurrence of myocardial ischemia in patients with atherosclerosis, the spleen provides an overabundance of inflammatory monocytes to atherosclerotic plaques throughout the arterial system, possibly promoting reinfarction.[6] Our data imply that GMI-1271 efficiently dampens inflammation in mice with atherosclerosis and MI, partially through reducing splenic production of myeloid cells.

Given that E-selectin has several other biological roles, the observed GMI-1271 effects are likely a consequence of several mechanisms, which may include inhibition of monocyte adhesion to endothelial cells. GMI-1271 also inhibits procoagulatory leukocyte action.[29] These 2 mechanisms, among others that are yet unknown, may have contributed to the observed reduction in plaque size and may be beneficial in the clinical setting.

Monocytes and macrophages are essential for efficient infarct healing. In wild-type mice with reduced leukocytes, for instance because of splenectomy or macrophage depletion, rupture rates are increased and post-MI remodeling leads to heart failure.[30,31] In patients, this scenario is rare because most of them have blood monocytosis and likely an overabundance of infarct macrophages. If there are too many inflammatory monocytes and macrophages residing in the infarct, resolution of inflammation is impaired, which also impedes infarct healing.[32] Thus, it has been postulated that there is an optimal range of monocyte supply to acute infarcts.[30] We

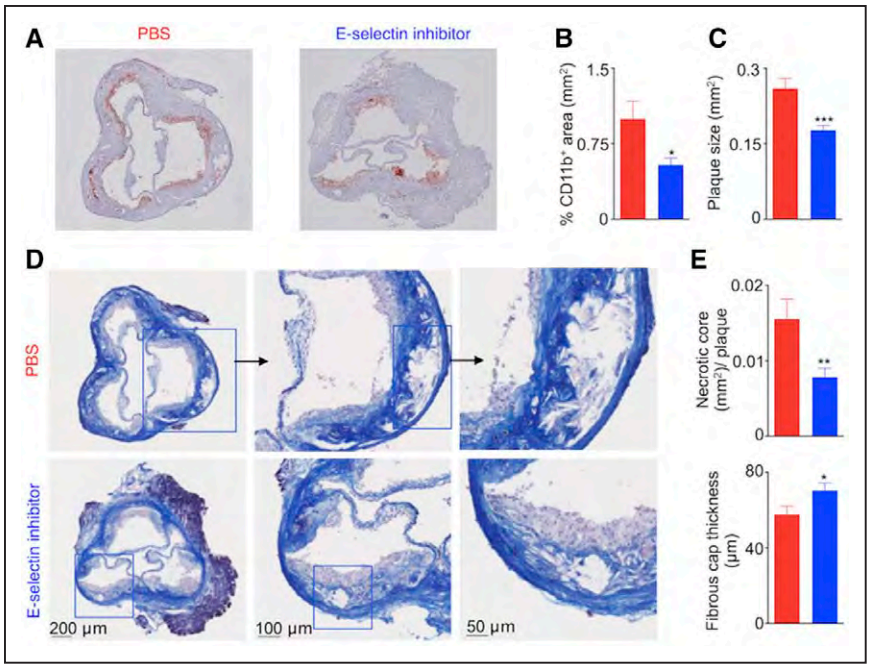

Figure 6. E-selectin inhibition improved features of stable atherosclerotic plaques. Representative images (**A**) and quantification (**B**) of CD11b⁺ area in aortic root sections. **C**, Quantification of plaque size after E-selectin inhibitor treatment. Representative images (**D**) and quantification (**E**) of necrotic core area and fibrous cap thickness in aortic root sections. Data are mean±SEM, n=9 per group, **P*<0.05, ***P*<0.01, ****P*<0.001.

therefore tested in wild-type mice whether GMI-1271 influences infarct healing. E-selectin inhibition reduced myeloid cell recruitment, but did not change scar size or left ventricular remodeling. These data indicate that GMI-1271 may have a favorable safety profile in patients with acute MI. Future experiments that focus on infarct healing in a setting of systemic monocyte/macrophage overabundance, for instance in mice with preexisting atherosclerosis,[32] will show whether anti-inflammatory properties of GMI-1271 support post-MI recovery of the left ventricle.

We did not observe an effect of GMI-1271 on bone marrow hematopoiesis in mice with atherosclerosis and MI. In steady state or after myelotoxic treatment, genetic deletion or E-selectin inhibition promotes HSC quiescence and survival.[13] We speculate that the bone marrow's state in mice with atherosclerosis and after MI may be altered and that these changes may interfere with drug distribution. An alternative explanation is that MI triggers strong and multifactorial hematopoiesis activation pathways, including via sympathetic nervous signaling[6] and circulating interleukin-1b,[33] which may override effects of E-selectin inhibition.

Our study has several limitations. We began to investigate splenic GMI-1271 effects in a worst-case scenario, a combination of acute (MI) and chronic (atherosclerosis) inflammation. Arguably, the clinical need for decisive but specific anti-inflammatory interventions is highest in this setting. Nevertheless, it is of interest whether similar drug action is observed in ApoE$^{-/-}$ mice without MI. Further, given the multiple biological functions of E-selectin, our data do not provide insight to what degree GMI-1271's splenic actions contributed to the observed phenotype. Thus, future studies should decipher the precise quantitative contribution of GMI-1271 inhibition of splenic cell production versus leukocyte migration. Finally, future studies should focus on the precise E-selectin ligand(s) in the spleen and reveal the identity of splenic cells involved in E-selectin-mediated information transfer.

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Disclosures

J. Magnani is an employee of GlycoMimetics and owns equity in the company. The other authors report no conflicts.

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Highlights

- An E-selectin inhibitor dampened splenic hematopoietic stem cell proliferation in mice with myocardial infarction and atherosclerosis.
- This treatment led to decreased splenic myeloid cell production and diminished supply of myeloid cells to atherosclerotic lesions.
- Ultimately, E-selectin inhibition resulted in a more stable plaque phenotype.

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E-Selectin Inhibition Mitigates Splenic HSC Activation and Myelopoiesis in Hypercholesterolemic Mice With Myocardial Infarction

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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

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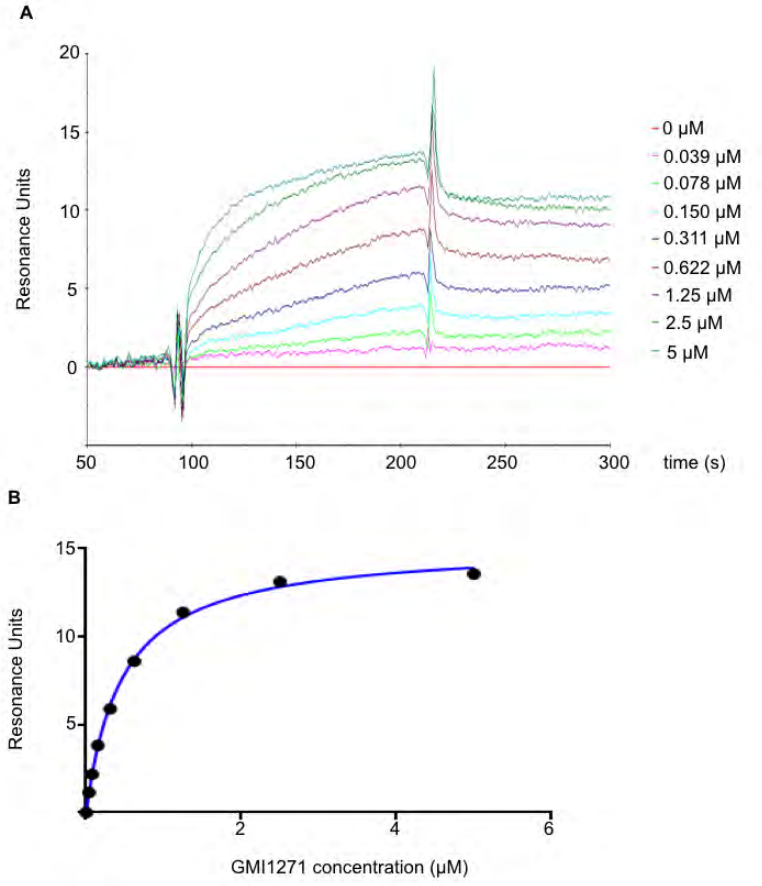
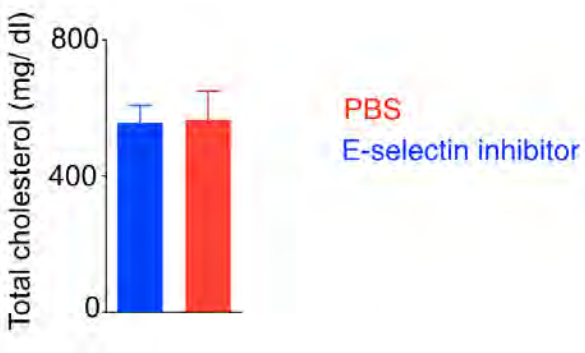
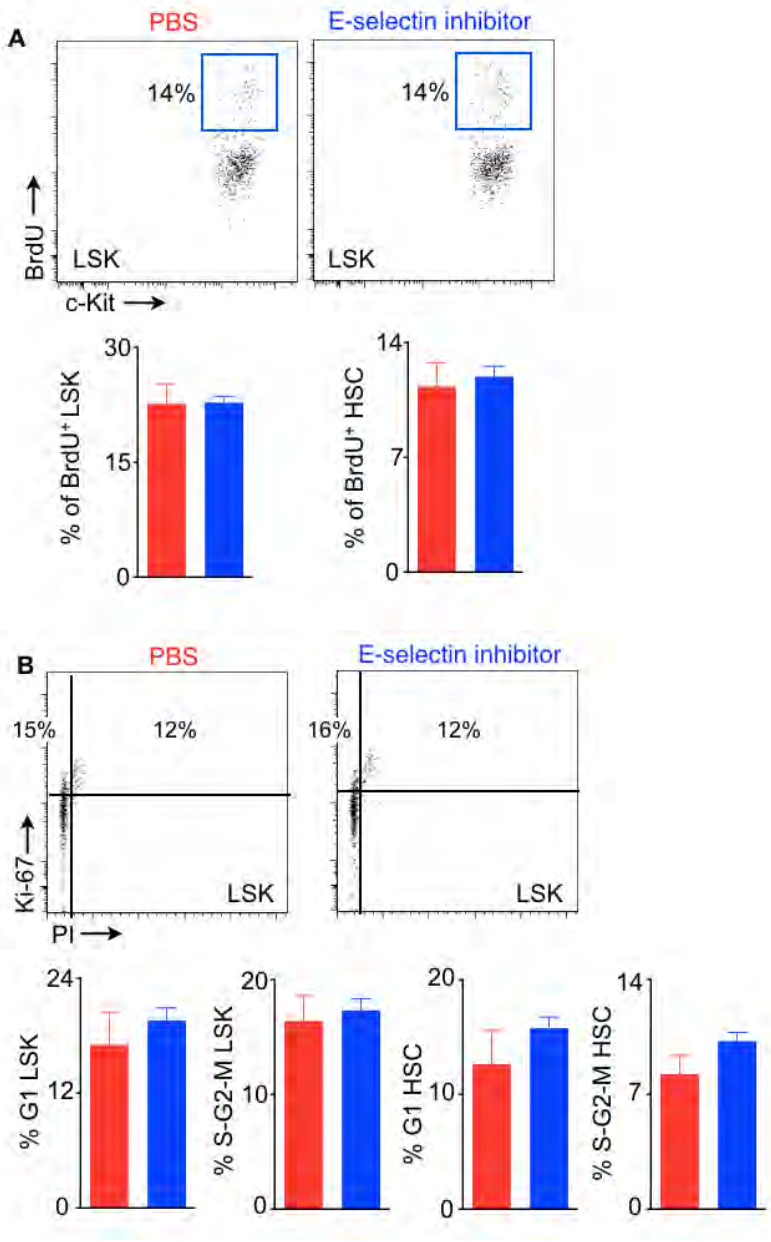
Supplemental figures



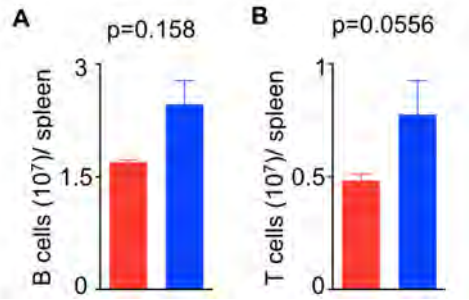
Supplemental figure I.
The dissociation constant K_D was assessed by means of surface plasmon resonance. Sensorgrams were recorded at different concentrations of GMI-1271 with a Biacore X100 instrument (A). The association, equilibrium and dissociation phases are expressed as resonance units over time. 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.



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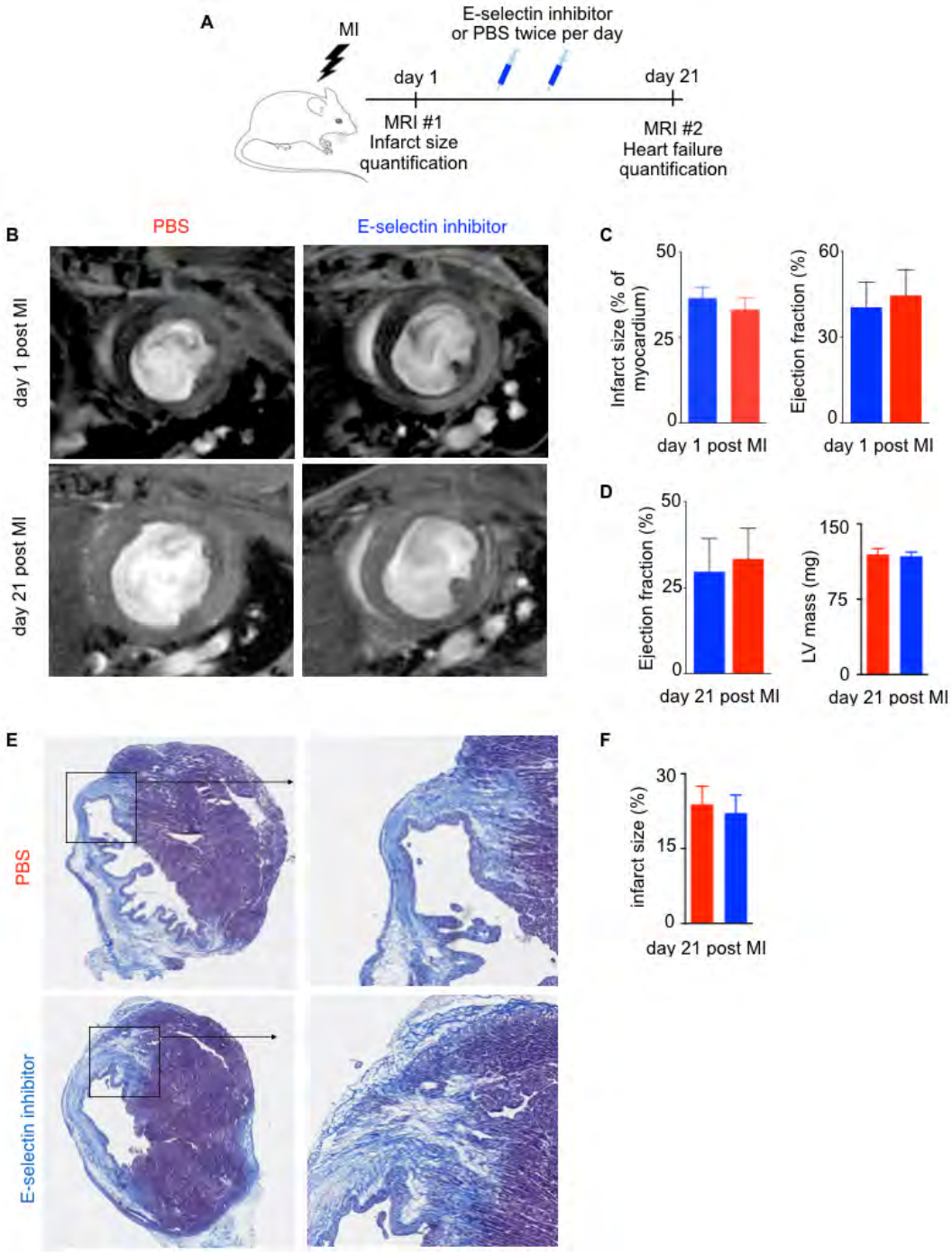
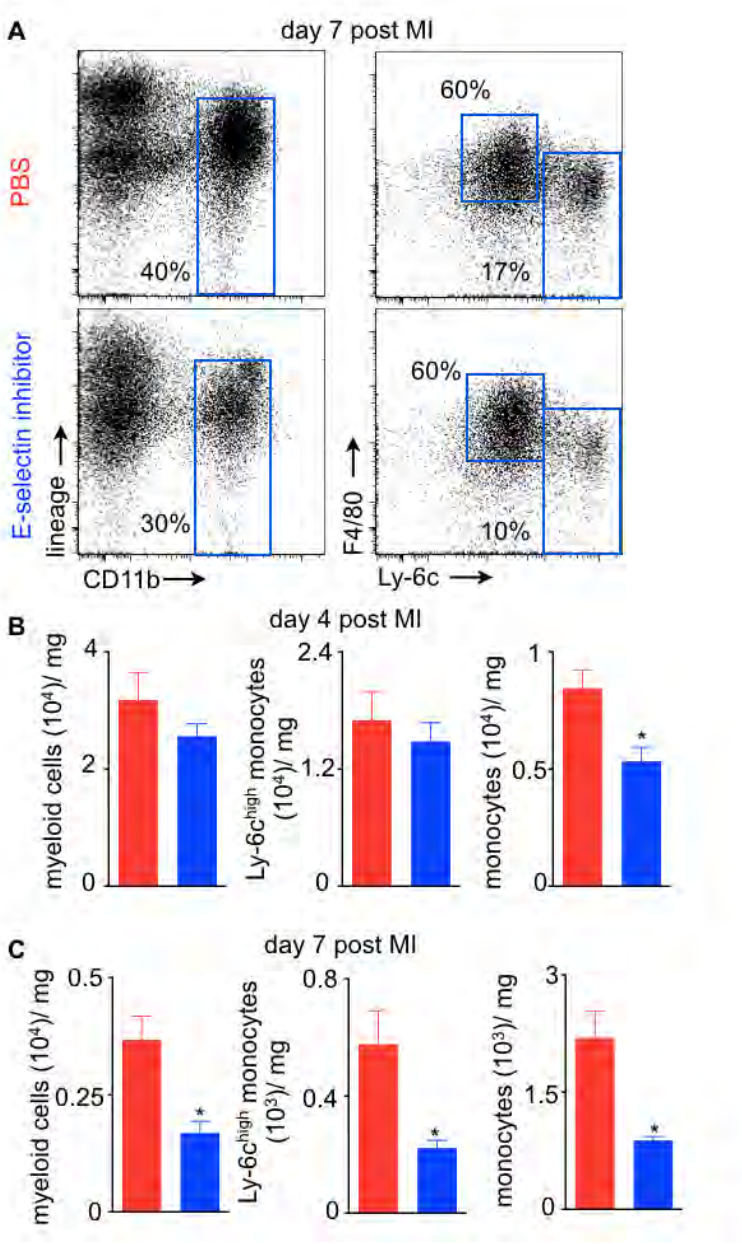
Lymphoid cells in the spleen were assessed by means of flow cytometry. While we observed a trend towards increased numbers of B-cells (**A**) and T-cells (**B**), the difference did not reach statistical significance. Data are mean ± SEM, n=5 per group.



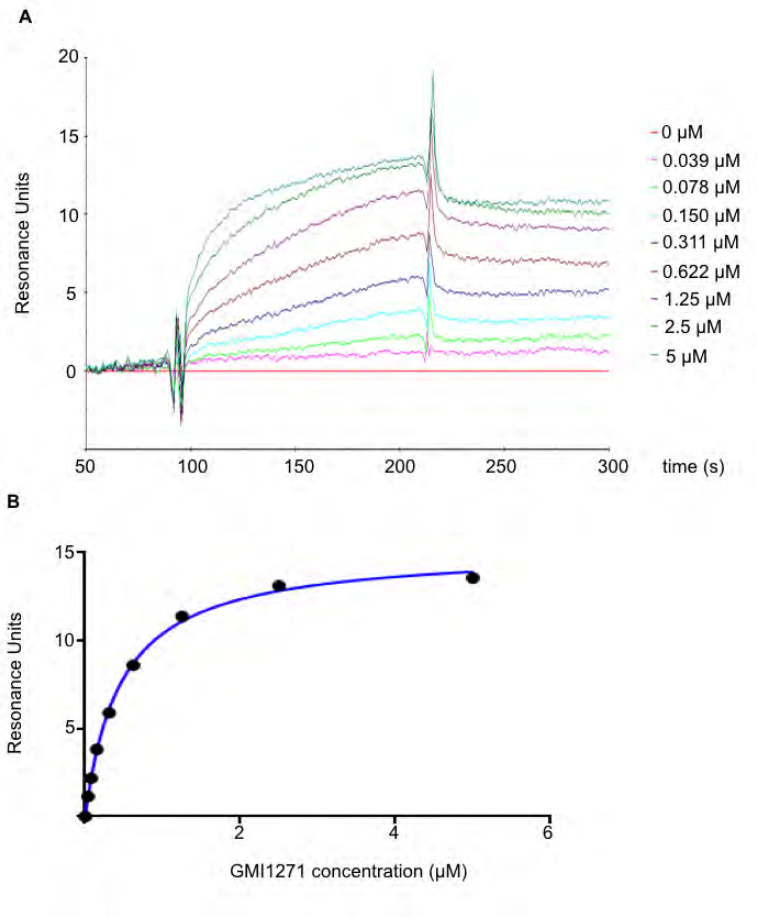
Supplemental figure V. E-selectin inhibition does not affect healing of the myocardium after myocardial ischemia. (**A**) Experimental plan. (**B**) Representative MRI short axis views. (**C**) Infarct size and ejection fraction (EF) measured by MRI on day 1 after MI. (**D**) EF and left ventricular (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.

SUPPLEMENTAL MATERIAL

Supplemental figures



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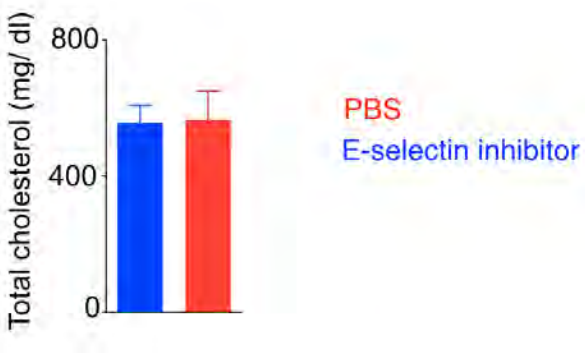

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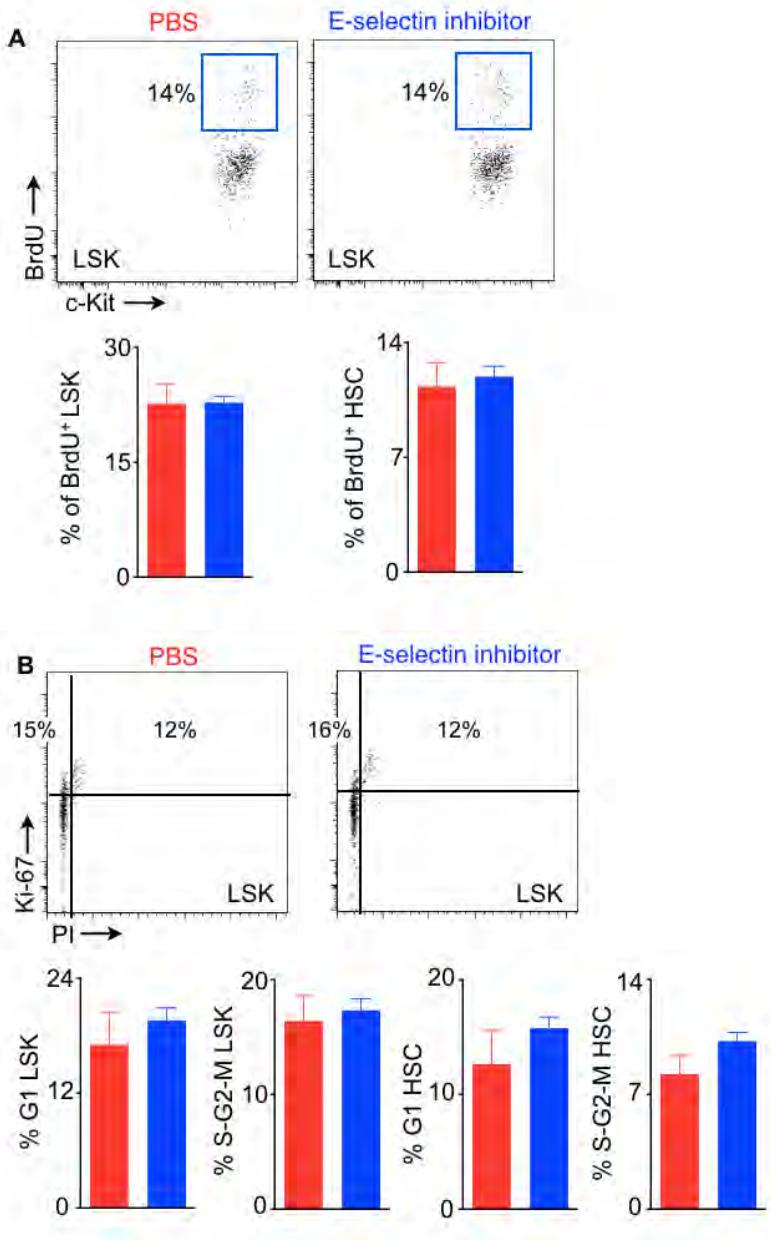

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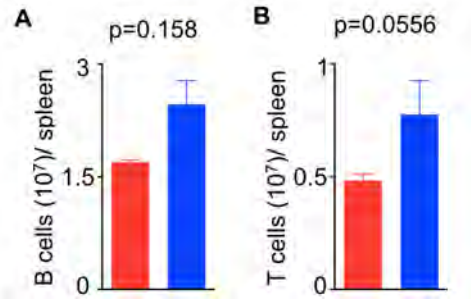

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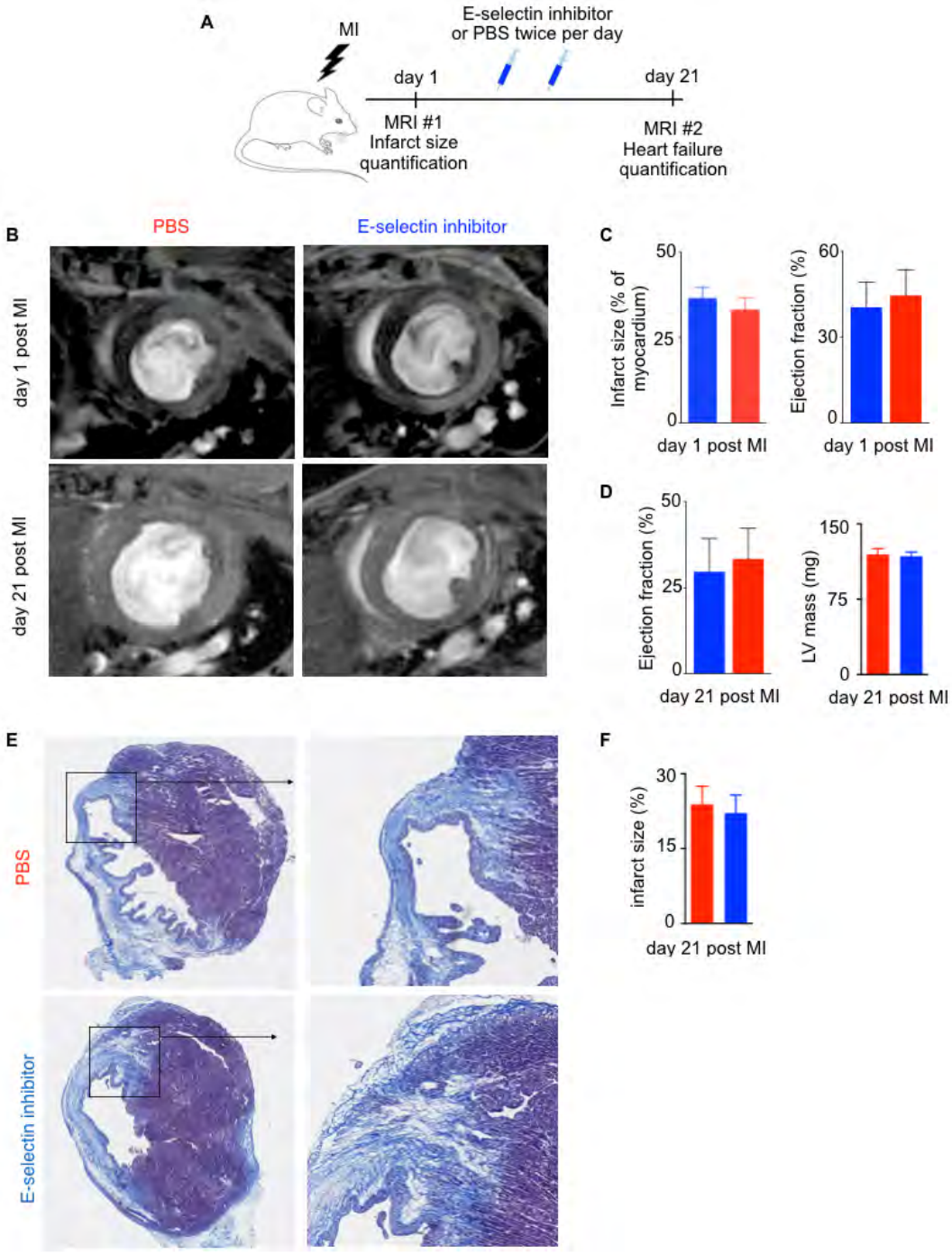

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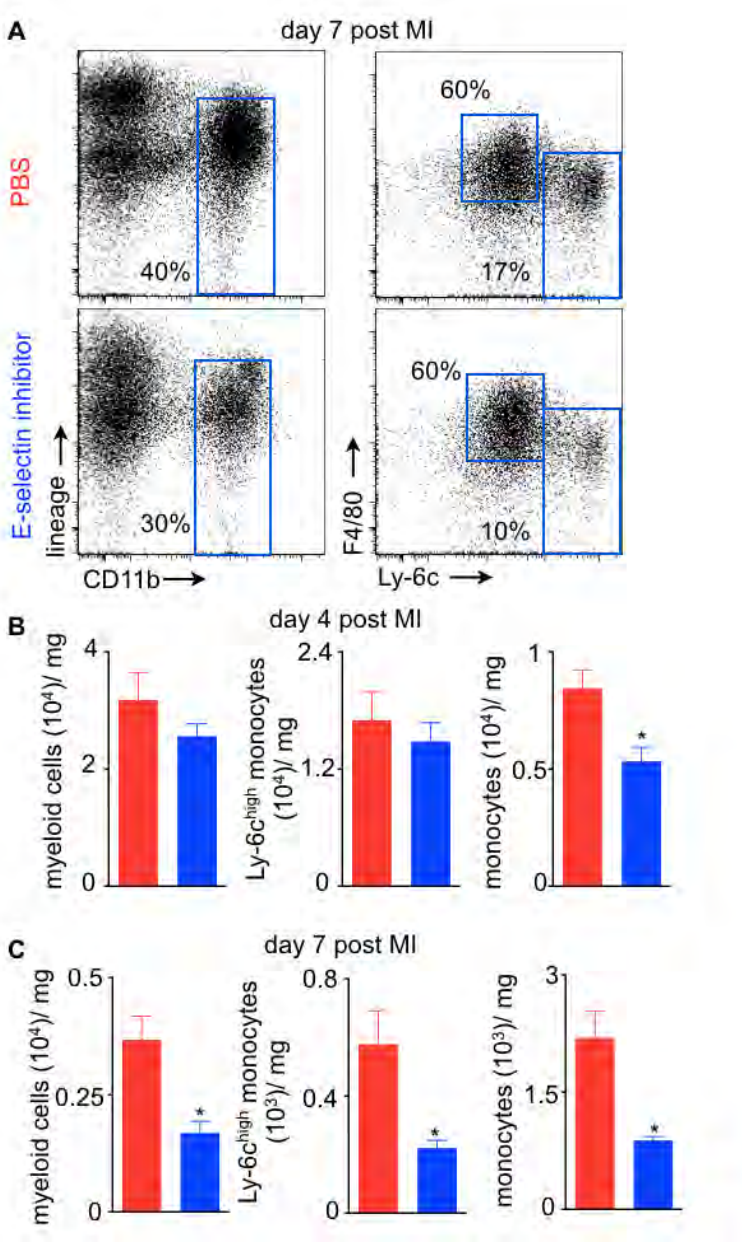

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