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Splenic release of platelets contributes to increased circulating platelet size and inflammation after myocardial infarction

[1,2*]Xiao-Ming Gao, [1,3*]Xiao-Lei Moore, [1*]Yang Liu, [4]Xin-Yu Wang, [1]Li-Ping Han, [1]Yidan Su, [1]Alan Tsai, [1]Qi Xu, [1,2]Gavin W. Lambert, [1]Helen Kiriazis, [4]Wei Gao, [12,,3]Anthony M. Dart, [1,2]Xiao-Jun Du

[1]Baker IDI Heart and Diabetes Institute, Melbourne, Australia; [2]Central Clinical School, Monash University, Melbourne, Australia; [3]Alfred Heart Centre, Alfred Hospital; [4]Department of Cardiology, Third Hospital of Peking University, Beijing, China.

*these authors contribute equally to this work.

YL's current address: Department of Cardiology, Institute of Cardiovascular Diseases, First Hospital of Dalian Medical University, Dalian, China.

Short running head: Splenic platelets and post-infarct inflammation

Correspondence: XJ Du, Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, Victoria 3004, Australia. T: 613-85321267; E-mail: xiao-jun.du@bakeridi.edu.au

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Abstract

Platelets contribute to the inflammatory response post myocardial infarction (MI), but the mechanism is partially understood. The spleen is known to store and releases monocytes and platelets. While mobilization of splenic monocytes contributes to post-MI inflammation, the role of splenic platelets is unclear. We determined mean platelet volume (MPV) and platelet-monocyte conjugation (PMC) using admission blood samples from patients with acute MI, as well as blood and the spleen from mice with MI by flow cytometry. Using immunohistochemistry, immunofluorescence and immunoblotting, we further measured changes in cardiac and splenic contents of platelets and leukocyte infiltration into the mouse infarct myocardium. In patients with MI, increase in both circulating MPV and PMC occurred within 3 h post-MI. Similar changes were observed in the blood and spleen in mice subjected to MI. At 24 h post-MI, splenic platelet storage was halved whereas cardiac accumulation of platelets increased by 4.4-fold. Splenectomy attenuated all changes observed in the blood, reduced leukocyte and platelet accumulation in the infarct myocardium, limited infarct size and alleviated cardiac dilatation and dysfunction. Acute MI was associated with elevated circulating levels of adenosine diphosphate and catecholamines, which may trigger splenic platelet release. Treatment with the angiotensin-converting enzyme inhibitor perindopril or β_1-adrenergic receptor antagonist atenolol suppressed increment of circulating platelet size or PMC as well as accumulation of platelets in the infarct myocardium. In conclusion, acute MI evokes release of splenic platelets, which contributes to the increase in platelet size and PMC and facilitates myocardial accumulation of platelets and leukocytes, thereby promoting post-infarct inflammation.

Key words:

platelets, spleen, inflammation, myocardial infarction, mean platelet volume, monocytes, catecholamines, infarct size

INTRODUCTION

In the setting of ischemic heart disease, significance of platelets is well known for their vascular actions involving promotion of atherosclerotic growth, plaque instability, thrombosis and restenosis following primary percutaneous coronary intervention (PCI) [1-3]. Clinical and experimental studies have provided evidence for pro-inflammatory action of platelets, independent of their actions of haemostasis and vascular thrombosis [3-7]. We previously documented activation of circulating monocytes in patients with acute myocardial infarction (MI) [8] and a pivotal role of circulating platelet-monocyte conjugation (PMC) and platelet accumulation in the infarct myocardium in a mouse model of MI [5, 9]. Recent findings have revealed that upon MI, there is a massive mobilization of splenic monocytes, particularly inflammatory M1-subtype ($CD16^-$ in humans and $Ly6C^{high}$ in mice), that later on infiltrate into the infarct myocardium [10-13]. Hence, the term "cardiosplenic axis" is used to highlight the splenic contribution to post-MI innate immune responses [10, 11, 14]. The spleen is also known to store (approximately 30% of total platelets), sequester and release platelets. To understand whether the concept of "cardiosplenic axis" extends to platelets is essential for development of a targeted anti-inflammatory strategy in treatment of MI.

Numerous clinical studies have revealed increase in circulating platelet size, measured as mean platelet volume (MPV), in patients with acute MI [15-23] and further bigger MPV is associated with increased risk of cardiovascular morbidity and mortality [15, 19, 22-24]. The mechanism for the enlarged circulating platelets, however, remains unclear. Newly generated platelets by megakaryocytes are usually bigger in size [25, 26]. The entire process of megakaryocyte differentiation and maturation needs 4-5 days, and a period of 24 h is required for de novo generation and release of platelets by mature megakaryocytes [25-27]. Thus, it is less likely that bone-marrow release of newly generated bigger platelets is responsible for this acute increase of MPV upon acute MI. Earlier studies showed that splenic platelets are 20-30% bigger and mobilization of splenic platelets could lead to increase in circulating MPV under stressed conditions including intense exercise and stimulation by cytokines or catecholamines [28-31].

We hypothesize that acute MI triggers a rapid mobilization of the splenic platelet pool, which is associated with the early increase in both size and activity of circulating platelets, contributing to systemic and cardiac inflammation. We studied human patients and mice with acute MI to assess platelet-related parameters in the blood, spleen and myocardium. Influences of splenectomy on these measures were determined in mice subjected to MI. Further, drug interventions were conducted to explore mechanisms of splenic platelet release and their subsequent accumulation in the infarct myocardium.

METHODS

Recruitment of human patients with acute MI

To explore temporal changes in circulating platelet size and PMC in patients with acute MI, we recruited patients with confirmed diagnosis of ST-elevation MI (STEMI) at Department of Cardiology, Third Hospital of Peking University, Beijing, China and at Alfred Heart Centre, the Alfred Hospital, Melbourne, Australia. Patients who received pre-treatment with anti-platelet drugs, except for aspirin, were excluded. These studies were approved by local clinical ethics committees. A written informed consent was obtained from all participants. STEMI was diagnosed according to the American College of Cardiology/American Heart Association guidelines in 2004. All patients received PCI and routine medications. Healthy subjects and patients with stable coronary artery disease (CAD) were also recruited for comparison. Blood samples were collected at admission prior to medication or interventional therapy for assays of MPV (Beijing cohort of patients) or PMC, adenosine diphosphate (ADP) and catecholamines (Melbourne cohort of patients).

Determination of platelet size, number and PMC in human patients

Venous blood samples were collected into a standardized vacutainer containing EDTA as anticoagulant. Routine haematological assay was performed within 30 min after blood collection using a Sysmex XE2100 Haematology System (Sysmex Corporation) at the Peking University Third Hospital. MPV and platelet count were measured.

For measurement of PMC, venous blood was collected from patients with MI at the time of admission (average 3 h after onset of MI) and from healthy volunteers or age- and gender-matched CAD patients. Peripheral blood mononuclear cells (PBMCs), isolated from fresh venous blood by Ficoll-gradient centrifugation [8], were used to quantify PMC by flow cytometry (FACS). In brief, PBMCs were incubated with antibodies against human CD62P (P-selectin, FITC-conjugated, BD Pharmingen) and CD16 (Percp-conjugated, BD Pharmingen) at 4^{o}C for 30 min in the dark, washed with PBS and then fixed in 2% paraformaldehyde. FACS was performed on BD FACSCalibur™ using appropriate settings excluding debris. 20,000 events per sample were collected within the monocyte gate. PMC results were expressed as percentage of $CD62P^{+}/CD16^{-}$ PMC over monocytes or $CD16^{-}$ monocytes (% of M1 monocytes) [5].

Animals and surgeries for coronary artery occlusion or splenectomy

Male C57Bl/6 mice (12-14 weeks of age) were used. All experimental procedures were approved by a local animal ethics committee and conformed to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013). Animals were anaesthetised with a mixture of ketamine/xylazine/atropine (100/20/1.2 mg/kg, i.p.). MI was induced by occlusion of the left coronary artery at the level 2 mm below the edge of the left atrium, as previously described [5]. For splenectomy, an abdominal incision was made to access the spleen. After occlusion of splenic arteries and veins, the spleen was removed and the abdominal incision closed by stitches in layers. To determine the influence of splenectomy in the infarct size, some animals were subjected to coronary artery occlusion for 1 h followed by reperfusion for 24 h [32]. Sham-surgery for coronary occlusion or splenectomy involved left thoractomy or abdominal incision, respectively. Abdominal surgery was performed immediately prior to thoracic surgery. Surgery-related loss of animals that occurred within 24 h was 5%. For comparison with splenectomy study, some normal mice were used as controls.

Echocardiography

Mice were anesthetized using isoflurane at 4.5% for induction and 1.7% for maintenance. Transthoracic echocardiography was performed using a Vevo 2100 ultrasound system (VisualSonics, Toronto, Canada) equipped with a 40 MHz linear array transducer (MS 550D). Animals were placed supine on an electrical heating pad at 37°C. Continual ECG monitoring was obtained via limb electrodes. Cardiac images of standard parasternal long-axis and short-axis were acquired. Analysis tool of the left ventricular (LV)-trace was used to outline the endocardium at the end-systole and end-diastole from optimal long-axis images. LV cross-sectional area and volume at the systole and diastole were measured and the fractional area change: [(area at diastole−area at systole) / area at diastole)] and ejection fraction (EF%): [(volume at diastole−volume at systole) / volume at diastole] were calculated. All parameters were obtained from >3 measurements and averaged using the offline software by experienced researchers.

Blood and tissue collection, organ weight and infarct size measurement in mice

Spleens and hearts were harvested at different time points after MI and weighed. The left LV was separated from right ventricle and atria, and infarct size was estimated as a ratio of either wet weight of infarct tissue to total LV mass or endosurface area, as we described previously [33, 34]. To ensure comparable effects of MI, animals with an infarct size less than 30% or over 55% of the LV was excluded. Tissues collected were either freshly processed or stored frozen till subsequent use. In experiments with splenectomy, infarct size was also determined at 24 h after ischemia-reperfusion injury. A dual staining method with 5% Evans blue and 1.5% triphenyltetrazolium (TTC) was used, as we described previously [32].

Platelet count in mice

At 6, 12 and 24 h post MI, together with control mice, blood samples (⩾20 μl) were collected from tail veins with Microvette® 200 containing EDTA were analyzed using Hemavet 950FS Blood Analyser (Drew Scientific) for platelet counting according to the manufacture's instruction.

Histology, Immunohistochemistry and Immunofluorescence

Intramyocardial haemorrhage. Heart tissues were fixed (10% buffered formaldehyde), paraffin embedded, and then sectioned at the thickness of 5 μm including the infarct region. Carstair's stain was performed to identify red blood cells and other structures in the myocardium, as we previously described [9]. Images were acquired using ImagePro software (Media Cybergenetics).

Platelet density in the heart and spleen. Hearts and spleens of mice were harvested at 48 h after MI and frozen sections were prepared. After blockade of mouse IgG using a commercial blocking MOM kit (Vector), sections were stained with rat-anti-mouse CD41 (GPIIb/IIIa) antibody (BD Biosciences), followed by secondary antibody, enzyme enhancer and permanent Red Chromogon. Mouse IgG isotype antibody (Jackson ImmunoResearch) was used as a control for non-specific signals. 4',6-diamidino-2-phenylindole (DAPI) was applied to stain nuclei and images acquired for analysis. Ten histological images were acquired per tissue sample and CD41-positive stained region was analysed digitally using ImagePro software and expressed as the percentage of imaging area [5].

Quantification of leukocyte density. Mouse heart frozen sections collected at 48 h post MI were stained with rat-anti-mouse CD45 antibody (BD Biosciences, USA), and followed by secondary antibody, Alexa Fluor® 546 goat anti-rat IgG (Life Technologies). Nuclei were

stained with ProLong® Gold antifade reagent with DAPI (Invitrogen). Multiple images (8-10 per LV) were acquired and CD45-positive leukocytes were counted manually and expressed as average cell number per mm^2, as we previously described [5].

Co-localization of platelets and leukocytes within the infarct myocardium. Freshly frozen hearts at 48 h post MI were used. Similar as above, after staining with rat-anti-mouse CD41 antibody and rat-anti-mouse CD45 antibody, secondary antibodies and DAPI were applied respectively and images acquired for analysis.

CD41 expression by Immunoblotting

Abundance of CD41 was determined in the spleen and heart tissues from mice with sham-operation or MI at 24 h after surgery by immunoblotting using anti-mouse CD41 antibody (BD Biosciences), as we previously described [5]. Results were expressed as ratio of house-keeping proteins (α-tubulin or total-AKT).

Flow cytometry (FACS) in animal studies

Platelet size, activation and PMC. To assess post-MI changes in platelet size and PMC, mouse blood was collected at 12 and 24 h after MI by cardiac puncture with heparin as anticoagulant. Platelet-rich plasma was prepared and 10,000 events per sample were collected within a platelet gate by BD FACSCalibur™. Forward-scattered light (FSC) of the collected platelets was used to indicate platelet size. For PMC measurement, red blood cell-lysed blood was incubated with corresponding antibodies (BD Biosciences) for 30 min at 4°C. A total of 20,000 events per sample were collected within a monocyte gate by BD FACSCalibur™, and analysed using the FlowJo software (Tree Star Inc). PMC was calculated as percentages of $CD41^+CD45^+$ PMC or $CD41^+CD115^+$ PMC over total monocytes [5]. In our earlier experiments, antibodies against CD41 and CD45 were used to identify PMC, and in subsequent experiments of drug intervention, antibodies against CD41 and CD115 were selected for more specific targeting at monocytes.

To determine the effects of splenectomy on changes in platelet size and PMC in circulation, platelet-rich plasma were prepared 24 h post-MI from MI mice with or without splenetomy. Platelets were incubated with rat-anti-mouse CD62P antibody (FITC-conjugated, BD Biosciences) and counted on BD FACSCalibur™. FSC was used as a measure for platelet size and mean FITC intensity (FL1) for activation levels of platelets [5]. PMC was measured as mentioned above.

Platelet conjugation with Ly-$6C^{high}$ leukocytes in mouse blood and spleen. To understand role of platelets in post-MI inflammation, we measured changes of platelet conjugation with Ly-$6C^{high}$ inflammatory leukocytes, in response to MI, both in the spleen and in blood. Blood and spleens were collected simultaneously from sham-operated mice and mice with MI at 1, 3 and 24 h after MI. A portion of the spleen was crushed in a FACS buffer (1% FBS/PBS with 2 mM EDTA) and cell suspension was filtered through a 40 μm cell strainer. After lysis of red blood cells, splenic cells were collected and resuspended in the FACS buffer, and centrifuged. Blood leukocytes were isolated from 50 μl blood following lysis of red blood cells and centrifugation. They were resuspended with the FACS buffer (100 μl). Both splenic cells and blood leukocytes were then incubated with anti-mouse Ly-6C (Percp-cy5.5.-conjugated, eBioscience) and CD41 antibody (FITC-conjugated, BD Bioscience) for 30 min at 4°C. Cells were thoroughly washed and then counted on BD FACSCanto™. 50,000 events per sample were collected within a monocyte gate. Analysis was performed using BD FACSDiva software (BD Biosciences).

Percentage of Ly-6C$^+$/CD41$^+$ cells over total monocyte counts was determined. Meanwhile, platelet size indicated by FSC in both circulation and spleen was also measured at 24 h post-MI.

Assays for adenosine diphosphate (ADP) or catecholamines in plasma

ADP levels in the plasma samples from patients with STEMI were determined using ADP-Glo™ Kinase Assay kit (Promega) following the supplier's instructions. This assay involves converting ADP into adenosine triphosphate (ATP) by a kinase reaction and then into light by Ultra-Glo™ Luciferase. Caution was given to prevent haemolysis of blood samples. Adrenaline and nonadrenaline of human or mouse plasma samples were extracted with activated alumina, separated by reverse-phase HPLC and quantified using an electro-chemical detector, as we previously described [35].

Determination of microvessel damage following MI

Microvascular damage occurs post-MI which might facilitate extravascular platelet accumulation and inflammatory cell infiltration in hearts. We therefore tested microvascular leakage using Evans blue as a permeability indicator. At 24 h post-MI, Evans blue (20 mg/kg, i.v.) was injected and 3 h later, animal was anesthetized. The aorta was cannulated for retrograde perfusion with saline to remove the dye in the vasculature. The LV was frozen on dry ice, sliced transversely into 6-7 slides (1 mm in thickness), and images were taken for identification of blue-stained zones indicating microvascular leakage. To quantify the leakage, tissue was minced, incubated with trichloroacetic acid (TCA, 50%) for 30 min and then homogenized using a metal-bead homogenizer (Bullet Blender^{TM}, USA). After centrifugation, supernatant (TCA extracts) was added into a 96-well plate in duplicates. Evans blue concentration was measured at 620 nm using a chromatographer (Bio-Rad, USA), calculated against standard curve and normalized by tissue weight.

Effects of angiotensin-converting enzyme (ACE) inhibitor, β_1-adrenergic receptor (β_1-AR) antagonist and platelet P2Y receptor inhibitor

To explore the mechanism of splenic platelets release following MI, we treated MI mice with, the ACE inhibitor perindopril (6 mg/kg), the β_1-AR antagonist atenolol (2.5 mg/kg), the platelet P2Y inhibitor clopidogrel (50 mg/kg loading dose, and 25 mg/kg maintenance dose) or vehicle (saline), respectively. A bolus intraperitoneal injection (i.p.) was given immediately before coronary artery occlusion followed by addition of perindopril or atenolol in drinking water or second bolus i.p. for clopidogrel. Twenty-four hours later, blood and spleen were collected for determination of platelet size (FSC) and PMC (% of CD115$^+$CD41$^+$ PMC over CD115$^+$ monocytes) by FACS and hearts for quantifying infiltrated platelet content by CD41 expression levels using immunoblotting.

Statistics

Results are expressed as mean±SEM, unless otherwise stated. Using GraphPad Prism software, data were analysed by ANOVA, and followed by multiple comparison test. $P<0.05$ was considered statistically significant.

RESULTS

Changes in platelet size and PMC in circulation post-MI

Platelet count and MPV were determined in 344 patients with STEMI and compared to that of healthy controls or patients with stable CAD. Table 1 shows basic clinical data of the three groups. Compared with healthy control group, patients with STEMI were older, had increased body mass index, higher incidences of hypertension, diabetes and hyperlipidaemia and were more likely smoking (Table 1). Moreover, MI patients had very low rates in pre-use of β-blocker, ACEI or ARB, asprin, and statins than stable CAD patients whilst the pre-use of aspirin was 89% (Table 1). Majority of these parameters were comparable between STEMI and CAD groups. In STEMI patients, at the time of admission, platelet count was not significantly different among the three groups. MPV, however, was considerably greater in STEMI versus the two control groups (Table 1). To trace a time-dependent change of MPV and platelet count, STEMI patients were re-grouped based on the time interval between onset of symptoms and blood sampling varying from 1 to 12 h (n=30~80 per group). STEMI patients showed a prompt increase in MPV by approximately 20% (*P<0.01*), as early as 1 h after onset of MI over control values (Figure 1A) but platelet count did not change (Figure 1B). PMC was increased by approximately 3-fold (*P<0.05*) at the time of admission (Figure 1C).

Similar to human patients, mice subjected to MI showed a significant elevation in both platelet size and PMC at 12 h and 24 h post-MI (Figure 1D, E), while platelet count was comparable to control mice during 6-24 h post-MI (Figure 1F). To test the hypothesis that splenic platelet release contributes to early increase in platelet size, we compared the platelets size isolated from blood and the spleen in mice without and with MI. We found that splenic platelet was approximately 25% larger than circulating platelets under normal condition (*P<0.01*, Figure 2A). At 24 h MI, the size of circulating and splenic platelets increased with splenic platelets remained bigger by 46% than circulating platelets (*P<0.01*, Figure 2B).

Reduction in the splenic platelet store post-MI

The spleen underwent a transient reduction in its mass during the period of 6 to 24 h after MI (Figure 2C). By 72 h post-MI, spleen mass returned to its normal level. Immunofluorescent staining (Figure 2D) of the spleen from normal control mice showed the presence of platelets ($CD41^+$ stained area) was largely at the subcapsular pulp and the marginal zone. Immunohistochemical staining of the spleen revealed that the $CD41^+$ stained region shrank by approximately 50% at 24 h after MI (*P<0.05*) versus that in control mice (Figure 2E). This finding was further confirmed by immunoblotting showing that at 24 h post-MI the CD41 expression in the spleen was significantly decreased by approximately 68% from the control value (*P<0.05*, Figure 2F). A partial depletion of splenic platelets was accompanied by a 4.4-fold increase in CD41 expression in the infarct myocardium (*P<0.05*, Figure 2G).

Circulating and splenic platelet-inflammatory leukocyte conjugation in mice post-MI

We then compared by FACS the degree of platelet conjugation to Ly-$6C^{high}$ cells in the blood and spleen at 1, 3 and 24 h after MI. Such conjugation increased by 1.5 to 2-fold (*P<0.05)* in the blood occurring as early as at 1 h after MI. The fraction of conjugation in the spleen increased by about 20% at 3 h (*P<0.05)* and further to 2.4-fold at 24 h over the baseline value (Figure 3A and 3B).

Effects of splenectomy on changes in platelet and inflammatory parameters, infarct size and ventricular remodeling

Splenectomy was applied to confirm the role of the spleen in the changes observed following MI. Blood was collected 24 h and hearts collected at 48 h post-MI in mice without and with splenectomy and normal mice served as the control. While MI induced a significant increase in all inflammatory parameters of blood and the infarct myocardium (Figure 4A-E), splenectomy significantly reduced platelet size by 15% (Figure 4A), activation of platelets by 7% (Figure 4B) and PMC by 32% (Figure 4C) in circulating blood (all *P<0.05*). At 48 h post-MI when myocardial infiltration of inflammatory cells reaches to its peak [5, 33, 34], splenectomy was associated with an approximately 24% reduction in platelet accumulation (*P<0.05,* Figure 4D) and furthermore a 32% decrease in leukocyte density in the infarct region (*P<0.05,* Figure 4E). At 48 h post-MI, echocardiography revealed in mice with splenectomy a reduced degree of LV dilatation and dysfunction in comparison to that of intact animals and a trend for a reduced infarct size (Table 2). In splenectomised mice subjected to ischemia-reperfusion, the infarct size was 55% smaller versus intact mice (*P<0.001,* Figure 4F), while area at risk was comparable (Figure 4F).

Elevated circulating levels of ADP and catecholamines following acute MI

In STEMI patients, plasma levels of noradrenaline were increased by 2-folds (*P<0.05,* Figure 5A) and that of adrenaline were tended to be higher than control values (*P=0.057*). The ratio of dihydroxyphenyglycol (DHPG)/noradrenaline, which is an indicator for neuronal reuptake function, was also significantly reduced in MI group (*P<0.05,* Figure 5A). Similar changes in plasma levels of noradrenaline and adrenaline in mice were also observed at 24 h post-MI (Figure 5B). In blood samples obtained from patients with acute MI, there was an 80% increase (*P<0.05)* in ADP levels at the time of admission but not at day-3 post-MI (Figure 5C).

Myocardial ischemia provoked microvessel damage

Following 24 h coronary artery occlusion, intramyocardial haemorrhage was detected within the infarct region by Carstair's staining (Figure 6B and 6C). Immunohistochemistry showed a close co-localization of platelets ($CD41^+$ staining) and infiltrated leukocytes cells ($CD45^+$ staining with dark stained nuclei) in the infarct myocardium at 48 h post-MI (Figure 6D). Furthermore, using Evans blue dye as an indicator, we detected a 26-fold (*P<0.001*) increased content of Evans blue in the infarct compared to non-infarct regions (Figure 6E) 24 h post-MI, suggesting microvascular hyper-permeability that might facilitates myocardial infiltration by circulating platelets and inflammatory leukocytes. Splenectomy had no effect on Evans blue content in the infract myocardium at 48 h post-MI when the severity of microvascular hyper-peameability further developed (Figure 6F).

Inhibition of ACE and β_1-AR attenuated platelet accumulation in the infarct myocardium

Acute MI is associated with elevation of circulating levels of angiogensin II [13] and catecholamines (Figure 5). To test whether these factors might regulate splenic platelet release, we treated mice with the ACE inhibitor, perindopril, or β_1-blocekr, atenolol, and measured changes in platelet size and PMC in circulating blood and CD41 abundance in the infarct myocardium. Treatment with perindopril largely abolished the increase of platelet size (*P<0.05,* Figure 7A) and reduced myocardial accumulation of platelets by 42% post-MI (*P<0.05,* Figure 7C), but had no effect on PMC (Figure 7B). Treatment with atenolol had no effect on platelet size post-MI, but significantly attenuated PMC by 37% (*P<0.05,* Figure 7B) and, similar to perindopril, reduced myocardial content of platelets by 63% (*P<0.05,* Figure 7D).

DISCUSSION

While increment in MPV in patients with acute MI has been well known, we observed this as early as 1 h after onset of MI. Meanwhile, PMC was elevated in patients within 3 h after MI. In mice, we confirmed that splenic platelets were larger than circulating platelets and that, similar to those in human patients, circulating platelets were bigger following acute MI. Importantly, splenectomy blunted this MI-induced increase in platelet size. These findings substantiate our hypothesis that enlarged platelet size is due at least in large part to splenic platelet release. We further observed in mice with MI that higher circulating and splenic PMC was accompanied by greater myocardial infiltration of platelets and leukocytes. Again, these changes were attenuated and infarct size was reduced by splenectomy, confirming a significant role of splenic platelet release in inflammatory response post-MI. Moreover, we demonstrated that β_1-AR or ACE inhibition effectively attenuated the MI-induced changes of circulating platelets and/or myocardial platelet accumulation, indicating that sympatho-β-AR and angiotensin II are involved in splenic release of platelets and platelet activation. Collectively, our findings demonstrate splenic platelets as a player in systemic and cardiac inflammatory response post-MI (Figure 8).

Increase in MPV at day-1 after acute MI has been reported by numerous clinical studies [15, 17-20]. In the present study, such change occurs in patients with MI as early as 1 h following symptom onset. Circulating platelets are heterogeneous in size, density and reactivity [25-27]. Large platelets are in general hyperactive in functionality, such as aggregation in response to collagen or ADP, release of thromboxane, and membrane levels of P-selectin or GP1b, GPIIb/IIIa [24-26]. Large-sized platelets also contain greater number of dense granules and hence granular release of active molecules in a larger quantity, including ADP and serotonin, but less responsive to anti-platelet drugs [18, 20, 23, 24, 36, 37]. Indeed, in patients with MI, bigger MPV is associated with increased risks of cardiovascular events [15, 19, 22, 24], acute stent thrombosis [18], or impaired reperfusion following PCI [17, 19, 21, 23, 24].

While newly generated platelets are usually larger in size [25-27], at least a 24-h period is required for their de novo generation and release by mature megakaryocytes upon stimuli such as cytokines [25, 38]. Studies have shown that splenic platelets are 20-30% bigger and hence splenic release of platelets is associated with increase in circulating MPV [28-31]. To address whether such early rise in platelet size is due to splenic release of stored platelets, we studied changes of platelet number and size both in blood and spleen and effects of splenectomy in mice before and after MI. Mouse splenic platelets are 25% larger than blood platelets and platelet size increased both in circulation and the spleen after MI. Reduced abundance of CD41 in the spleen implied a partial depletion of platelet storage following MI. Splenectomy blunted all these changes induced by MI. These findings support our hypothesis that splenic platelets release into circulation following acute MI resulting in early rise of MPV. Importantly, reduction of splenic platelet storage is accompanied by an increase in the platelet content in the infarct myocardium following MI. Such reciprocal changes imply that splenic platelets are a major source of extravascular accumulated platelets in the infarct myocardium.

Clinical and experimental studies have shown augmented circulating PMC following MI [5, 6, 39]. A new finding from the present study is that PMC also increased in the spleen following MI. Formation of PMC is a pivotal process by which platelets "inflame" leukocytes [3, 5, 7]. Swirski *et al* reported that splenic release of Ly-6C^{high} monocytes is coupled with infiltration of infarct myocardium by these cells [10, 13]. The term "cardiosplenic axis" is then used to highlight the contribution of the spleen to post-MI innate immune response [10-14]. It remains

unknown whether release of splenic platelets occurs in a similar fashion, albeit the spleen is known to store approximately 30-40% of total platelets [27, 40, 41]. Here we provided experimental evidence for association between an increased percentage of PMC in both spleen and blood and a higher cardiac co-localization of infiltrated platelets and leukocytes post-MI, a dynamic process similar to that of splenic inflammatory (M1) monocytes. Thus, splenic PMC is expected to contribute to activation and splenic release of inflammatory monocytes into the circulation upon acute MI (Figure 8) [11-14].

All MI-induced platelet and inflammatory changes seen in the circulating blood or the infarct myocardium were attenuated in mice with splenectomy, highlighting a significant role of the splenic monocytes/platelets in post-infarct systemic and cardiac inflammation. According to the previous studies documenting splenic release of pro-inflammatory monocytes upon MI [10-14], splenectomy would be expected to attenuate inflammatory response by removal of the splenic pool of Ly-6C^{high} monocytes. Indeed, splenectomy attenuated inflammatory cell infiltration in the infarct myocardium, reduced circulating platelet size, fraction of activated platelets and PMC, and notably, decreased platelet accumulation in the infarct myocardium. Consequently, splenectomy decreased infarct size and attenuated LV chamber dilatation and dysfunction in the acute phase. These results underscore the splenic mobilization of monoytes/platelets as a major contributor to the systemic inflammation following ischemic insult. These findings also extend the current understanding of inflammatory property of platelets emphasizing the role of splenic platelet pool in contributing to activation of circulating leukocytes (Figure 8), particularly inflammatory monocytes [5, 10, 13, 14, 42]. Our findings support the significance of "cardiosplenic axis" in post-MI inflammation and further enrich the content of such cardiosplenic coupling by demonstrating release of platelets as a pivotal part of the pathophysiology of stress and inflammatory responses following MI (Figure 8).

It remains unclear on exactly how splenic platelet release is initiated and how platelets are activated. In patients or mice with acute MI, circulating levels of catecholamines increased 2-3 folds. This finding may provide clues for mechanism triggering splenic platelet release. Clinical studies have also suggested that conditions associated with sympatho-β-adrenergic activation, such as intense exercise or administration of catecholamines, induce splenic release of platelets likely mediated by β-adrenergic activation [28-31, 40, 43]. In addition, Swirski *et al* provided evidence for elevated levels of circulating angiotensin II in mediating splenic release of monocytes following acute MI [13]. Thus, elevated levels of angiotensin II and catecholamines may jointly promote splenic platelet release. Encouragingly, treatment with perindopril or atenolol in mice for 24 h commencing at the time of coronary artery occlusion significantly reduced circulating platelet size, PMC and platelet accumulation in the heart, indicating the involvement of elevated circulating levels of angiotensin II and catecholamine post-MI in splenic release and activation of platelets. We compared MPV in patients with or without pre-use of β-blocker, ACEI/ARB, aspirin or statins (Table 1), and found lack of significant difference in MPV by pre-use of these drugs (data not shown). Our data, however, is limited by insufficient sample sizes (Table 1). Further prospective study with adequate sample size is required for a conclusive answer.

In keeping with a previous report [44], we observed in patients within 3-4 h after MI, a 80% increase in circulating levels of ADP, which is able to activate platelets via P2Y receptors. Notably, our previous study in mice showed that blockade of ADP receptor P2Y by clopidogrel and prasugrel inhibited platelet activation measured by P-selectin expression post-MI [5, 9]. Recent studies have shown that myocardial necrosis post-MI evokes release of a variety of molecules termed damage-associated molecular patterns (DAMPs) [45]. Through binding to

pattern recognition receptors such as Toll-like receptor (TLR), DAMPs activate innate immunity as well as platelets [45, 46]. Indeed, Semerato *et al* elegantly showed extracellular histones as the potent activator of platelets through TLRs [46]. Thus, it is likely that following MI these factors synergistically trigger splenic release of platelets/monocytes, activate splenic and circulating platelets and monocytes, and promote PMC (Figure 8), thereby contributing to systemic and regional inflammation.

Two possibilities may explain the extravascular platelet accumulation in the infarct region: 1) a "piggy back" mechanism by which leukocytes conjugated with platelets migrate into the infarct region, and 2) microvascular damage allowing platelets to enter into myocardial interstitium. Our previous [5] and the current study have provided histological evidence for co-existence of platelets and leukocytes within the infarct myocardium. Further we showed microvascular hyper-permeability in the infarct myocardium, evidenced by intra-myocardial haemorrhage of extravascular localization of red blood cells and severe microvascular hyper-permeability in the infarct region. Thus, microvascular damage is critical in facilitating migration of circulating platelets and inflammatory cells into the infarct region. While having no effect on the severity of microvascular damage post-MI, splenectomy reduced accumulation of inflammatory cells and platelets in the infarct myocardium, implying that this effect is not due to change of microvascular hyper-permeability per se, but due to reduced number and activity of spleen-derived monocytes and platelets.

In conclusion, our study provides novel findings for the role of splenic release of platelets as well as monocytes, in the early phase of MI, which have clinical implications. *First*, we document a mechanism responsible for the rapid increase in circulating MPV in patients shortly after MI. *Second*, our finding highlights the significance of splenic platelets/monocytes in promoting inflammatory responses upon onset of MI. We illustrate a chain of events from splenic platelet release, increase in size and activity of circulating platelets, increase in PMC in both peripheral blood and the spleen, to myocardial accumulation of platelets and leukocytes. *Third*, splenectomy attenuated the sequential changes of systemic and regional inflammation and consequently, reduced infarct size and alleviated ventricular remodeling and dysfunction at the acute phase following ischemic insult. *Forth*, therapies targeting β_1-AR and ACE attenuate the MI-induced changes of circulating platelet and platelet accumulation in the infarct myocardium. Thus, the spleen acts as a key organ in the systemic response to acute ischemic injury by releasing monocytes and platelets to promote inflammation, and platelets are another important player in the "cardiosplenic axis" (Figure 8).

Previous studies have documented splenic release of monocytes upon MI contributing to the systemic and regional innate immune response [11-14] . While we demonstrated in the present study the splenic release of platelets, this finding is not inconsistent with these studies on the significance of splenic monocytes, rather to delineate a role of co-released platelets in promoting inflammatory response. This is especially the case considering the strong evidence from previous clinical and experimental studies that PMC is a major mechanism for monocyte activation [3-6]. In the present study, we demonstrated the splenic platelet release as a mechanism responsible for the enlarged platelet size in acute MI. Platelet size, however, is an indirect index associated with platelet activity [36] . Previous studies, including ours, have shown that acute MI triggers platelet activation measured by P-selectin positivity, PMC and myocardial platelet infiltration [4-6, 9] . Platelets have been implicated in mediating myocardial damage and cardiac remodelling (e.g. regional inflammation, wall rupture, LV thrombus), which were attenuated by anti-platelet interventions (experimental thrombocytopenia, P2Y inhibitors) in the mouse model of MI [5, 9]. In the present study,

cardiac protection was also achieved by splenectomy measured as inflammatory infiltration and infarct size. Our findings highlight the importance of the splenic co-release and interaction of monocyte/platelets in mediating systemic and regional inflammation, which forms a potential therapeutic target.

Clinical perspectives

The inflammatory property of platelets, largely through interaction with leukocytes, has been well appreciated. Clinical studies commonly reported increment of circulating platelet size following acute MI. The reason for this change and its relation to cardiac inflammation post-MI remains unclear. Using the mouse model of MI, we demonstrated mobilization of splenic platelet store that is related to increased platelet-bound inflammatory monocytes in circulation and myocardial infiltration by inflammatory cell as well as platelets. Importantly, splenectomy, inhibition of AEC or β-AR abolished or attenuated increase in circulating platelet size and the extent of cardiac inflammation. Furthermore, splenectomy limited the infarct size and alleviated cardiac remodelling at the acute phase. Our study provides a mechanism for the acute increase in circulating platelet size in patients with acute MI by demonstrating mobilization of the splenic platelet/monocyte store. The spleen is a key organ in mediating systemic and cardiac inflammation through release of platelets and monocytes upon acute MI.

Author contribution

XM Gao, XL Moore, Y Liu: contributed to concept and study design, data generation, analysis and interpretation of data, and critical writing or revising the intellectual content. LP Han, Y Su, A Tsai, Q Xu, H Kiriazis, XY Wang, G Lambert: contributed to data generation. W Gao, AM Dart, XJ Du: contributed to concept, study design and research fund, data generation, analysis and interpretation of data, critical writing the intellectual content. All authors have approved the final version and this submission.

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Summary statement

We demonstrated splenic release of platelets as a mechanism for early enlargement in circulating platelet size observed in patients or in mice with acute myocardial infarction. This process, together with monocyte release, contributes to systemic and myocardial inflammation, infarct size and dysfunction following acute myocardial infarction.

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Table 1. Basic clinical and haematological parameters in healthy subjects and patients with stable coronary artery disease (CAD) or acute myocardial infarction (MI).

	Healthy control	Stable CAD	Acute MI
Number	55	25	344
Male gender (%)	65	52	80†
Age (year)	54 ±10	62 ±9*	61 ±13*
Diabetes (%)	0	16	35*
Hypertension (%)	0	76*	52*
Hyperlipidaemia (%)	0	48*	37*
Current smoking (%)	16	32	49*
Body mass index (kg/m^2)	22.5 ±2.5	25.9 ±3.6*	24.0 ±2.4*
Platelet count (10^9/L)	203±41	222±66	221±59
Mean platelet volume (fL)	8.6±0.9	8.4±1.1	10.1±1.0*†
Drug pre-use			
Beta-blocker	0	84%*	6.1%$_{\dagger}$
ACEI or ARB	0	72%*	9.9%†
Asprin	0	92%*	89%*
Statins	0	88%	5.8%†

Data are mean± SD. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker. *P<0.05 vs. healthy control, $^{\dagger}P$<0.05 vs. stable CAD.

Table 2. Influence of splenectomy in left ventricular (LV) remodelling and function at 48 h after MI.

	Heart rate (beats/min)	**LV area-systole** (mm^2)	**LV area-diastole** (mm^2)	**Fractional area change** (%)	**LV volume-systole** (μl)	**LV volume-diastole** (μl)	**Ejection fraction** (%)	**Infarct size** (%)
Sham *(n=15)*	436±10	15.3±0.7	23.8±0.8	36±1	34±5	64±4	50.1±1.9	–
MI, intact *(n=8)*	546±20*	20.6±1.2*	26.1±1.3*	21±2*	57±5*	78±6*	23.0±2.8*	42.7±3.5
MI, splenectomy *(n=8)*	560±18*	17.2±1.4*†	25.0±0.9*	32±4†	41±6†	73±5	44.5±5.4†	35.6±3.3

Data are mean±SE. MI, myocardial infarction. *$P<0.01$ vs. sham, $^{\dagger}P<0.01$ vs. MI, intact group.

FIGURE LEGENDS

Figure 1. Changes in platelet size and platelet-monocyte conjugation (PMC) in human patients or mice following acute myocardial infarction (MI).
A-B, Changes in human patients of mean platelet volume (MPV, A) and platelet count (PLC, B) at different interval following onset of symptom. Patients (n=344+24h+72h) were re-grouped (n=38~158) according the time-interval between onset of symptoms to blood sampling. Results from healthy subjects (n=55) and age- and gender-matched patients with CAD (n=25) are presented as reference. Results are mean±SD. *$P<0.01$. The green arrow indicates commencement of medication. **C,** Representative FACS plots showing increased PMC in patients with acute MI (n=7/group) with results presented as PMC percentage of $CD16^-$ cells (M1 monocytes). Grouped data depicted a temporal change of non-inflammatory ($CD16^+$) and M1 monocyes ($CD16^-$) in the circulation post-MI. Results are mean±SD. *P<0.05 vs. the same cell type in control (CTL), †P<0.05 vs. 3h $CD16^-$. The green arrow indicates commencement of medication. **D-F,** Changes in platelet size (D), PMC (E) and platelet count (F) of mice at different time points after coronary artery occlusion. Results are mean±SEM, n=7-9/group. *P<0.05 vs. CTL.

Figure 2. Changes in platelet size, spleen weight and tissue platelet content in mice after myocardial infarction (MI).
A-B, Splenic platelets were larger than blood platelets in normal mice (A) and this difference was augmented in mice post-MI (B). *$P<0.01$. **C**, Transient reduction in the spleen weight normalized by body weight during 6 to 24 h after MI. n= 8-12 per MI group and n=18 for control group. **D,** Immunofluroscence (CD41) staining showing platelet-positive stain (red) in the marginal zone and subcapsular red pulp of the spleen from normal mice. Blue stain is for nuclei by DAPI. **E,** Representative immunohistochemistry showing reduction in the abundance of splenic platelets (red) at 24 h post-MI versus sham-operated mice. **F,** Reduction in platelet content determined by CD41 abundance by immunoblotting in the spleen 24 h after MI versus control mice (n=6/group). **G,** Reciprocal increase in CD41 expression by immunoblotting in the infarct myocardium at 24 h post-MI (n=5-6/group). *P<0.05 vs. control values (C-G).

Figure 3. Temporal changes of platelet-leukocyte conjugation in the spleen and blood in mice after myocardial infarction (MI).
A, Representative FACS plots displaying changes in platelet and inflammatory leukocyte conjugation determined by the population of cells expressing high levels of CD41 and Ly-6C in the spleen and blood samples from mice at baseline or at 1, 3 and 24 h following MI. Quantitative data are presented in panel **B,** n=5-7/group. *P<0.05 vs. baseline values.

Figure 4. Effects of splenectomy on platelet and inflammatory parameters in mice with acute myocardial infarction (MI) and the infarct size.
A-C, Compared with intact mice, splenectomy significantly reduced circulating blood platelet size (A), platelet activation measured by $CD62P^+$ platelets (P-selectin, B) and PMC measured as $CD45^+/CD41^+$ monocytes over total monocytes analysed by FACS (C) at 24 h post-MI. *$P<0.05$. **D-E**, In the infarct myocardium, plenectomy notably reduced platelet accumulation ($CD41^+$ stained area, red colour, D) and leukocyte infiltration ($CD45^+$ stained cells showing pink with blue DAPI stained nuclei, E) at 48 h post-MI versus intact mice. *$P<0.05$. **F**.

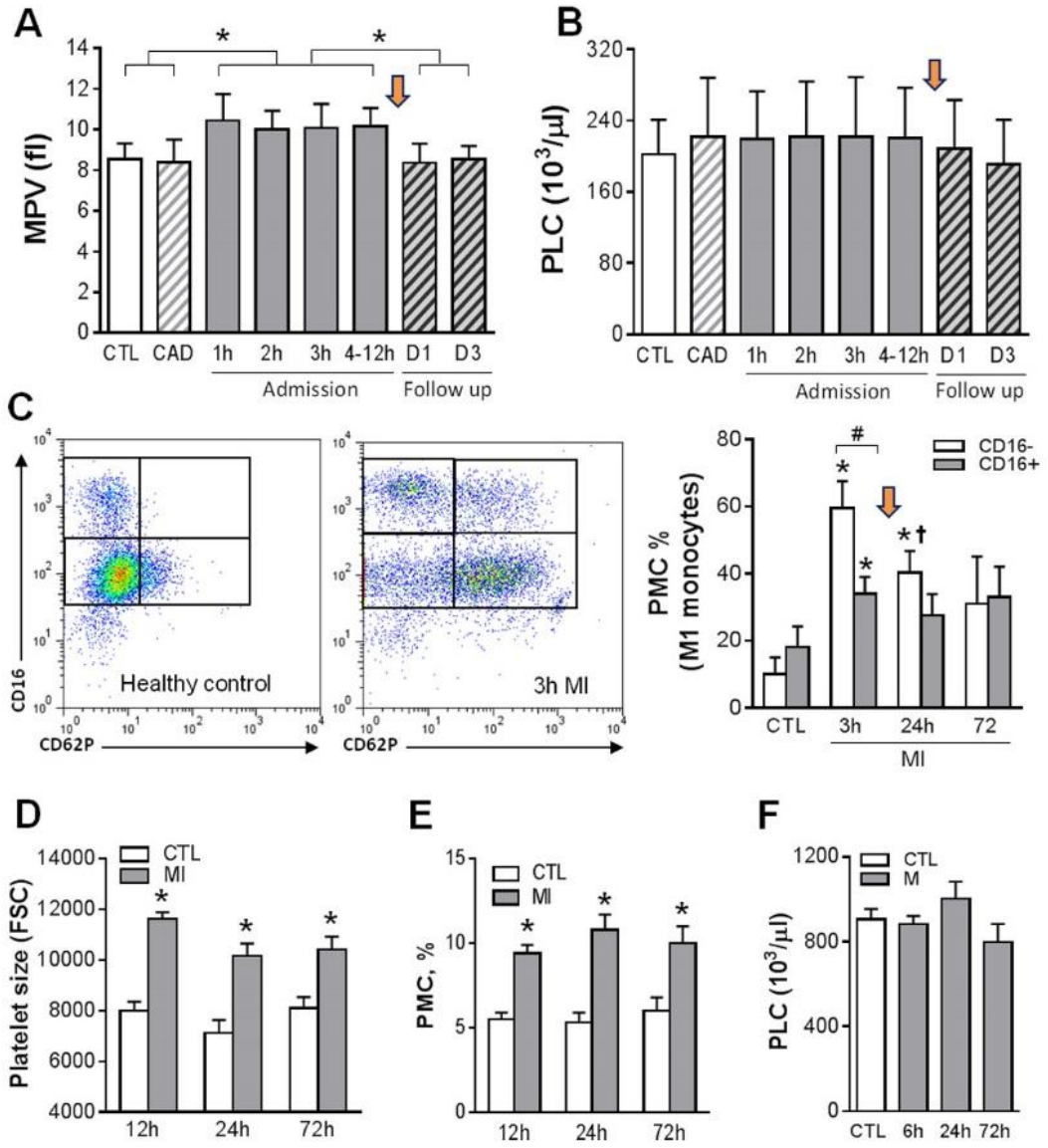
Splenectomy reduced infarct size at 24 h following ischemia-reperfusion (IR) with a comparable extent of ischemic insult measured as the area at risk (AAR). n= 9-10/group. *$P<0.001$ vs. intact mice (+spleen).

Figure 5. **Increased plasma levels of catecholamines and adenosine diphosphate (ADP) in patients or mice with acute myocardial infarction (MI).**
A, Elevated plasma levels of noradrenaline (NA) and adrenaline, and decreased neuronal reuptake activity indicated by the lower ratio of dihydroxyphenyglycol (DHPG)/NA in patients at day-1 after MI (n=14) in comparison with that of age- and gender-matched healthy controls (n=9). **B,** Elevated plasma levels of NA and adrenaline in mice at 24 h post-MI. n=4-6/group. **C,** Elevated plasma levels of ADP in patients with acute MI at the time of admission (3-5 h post-MI) but not at day-3. *P<0.05 vs. control, $^{\#}P<0.05$ vs. day-3 MI values.

Figure 6. Intramyocardial haemorrhage and microvascular hyperpermeability in hearts of mice with acute myocardial infarction (MI).
A-C, Carstair's staining of mouse hearts with sham-surgery (A) or with MI for 24 h (B-C) depicting extravascular presence of red blood cells (orange stained, black arrows) and thrombus (blue arrow). **D**, Immunohistochemical staining displays a co-localization of platelets (CD41-positive stained red area) and infiltrated inflammatory cells (dark stained nuclei). **E**, Representative left ventricular sections showing a leakage of Evans blue dye in the infarct segments, and quantitative data indicate a marked elevation of Evans blue content in the infarct zone (IZ). n=4/group. *$P<0.001$ *vs.* non-infarct zone (NIZ). **F.** Splenectomy did not affect Evans blue content in the infarct myocardium. n=7-8/group.

Figure 7. Effects of pharmacological inhibition of angiotensin-converting enzyme (ACE), β_1-adrenergic receptor and platelets on platelet and inflammatory parameters in mice with myocardial infarction (MI).
A-B, treatment with AEC inhibitor perindopril blunted the increase of circulating platelet size (A) while treatment with the β_1-antagonist atenolol partially and with clopidogrel completely reduced PMC in circulating blood (B) at 24 h post-MI. **C-E,** treatment with perindopril (C), atenolol (D) or clopidogrel (E) attenuated CD41 expression (platelet accumulation) in the heart at 24 h post-MI. n=5-8/group. *$P<0.05$ vs. normal (A, B) or vehicle (C-D) group, $^{\#}P<0.05$ vs. vehicle group (A, B).

Figure 8. Schema depicting the potential role of splenic platelets in inflammatory response after acute myocardial infarction (MI).
Acute MI rapidly induces a cascade of neurohormonal activation measured by increased levels of catecholamines and angiotensin II and also evokes release of danger associated molecular patterns (DAMPs) and adenosine diphosphate (ADP) from ischemic tissues (**A**) [45, 46]. These factors mediate platelet activation and platelet-monocyte interaction ($\leftrightarrow$) occurring within the spleen and in the circulation (**B**). Increased circulating levels of catecholamines and/or angiotensin II [13, 14] trigger rapid splenic release of platelets into the circulation and platelet-monocyte conjugation (PMC) (**C**), a process coinciding with splenic release of monocytes as documented previously by other groups [13, 14]. Activated circulating inflammatory cells and platelets then infiltrate into the jeopardised myocardium promoting regional inflammation with subsequent exacerbation of myocardial injury (**D**).


A
MPV (fl)
CTL CAD 1h 2h 3h 4-12h D1 D3
Admission
Follow up
B
PLC (10^3/µl)
CTL CAD 1h 2h 3h 4-12h D1 D3
Admission
Follow up
C
CD16
CD62P
Healthy control
3h MI
PMC % (M1 monocytes)
CD16-
CD16+
CTL 3h 24h 72
MI
D
Platelet size (FSC)
CTL
MI
12h 24h 72h
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PMC, %
CTL
MI
12h 24h 72h
F
PLC (10^3/µl)
CTL
M
CTL 6h 24h 72h

Figure 1

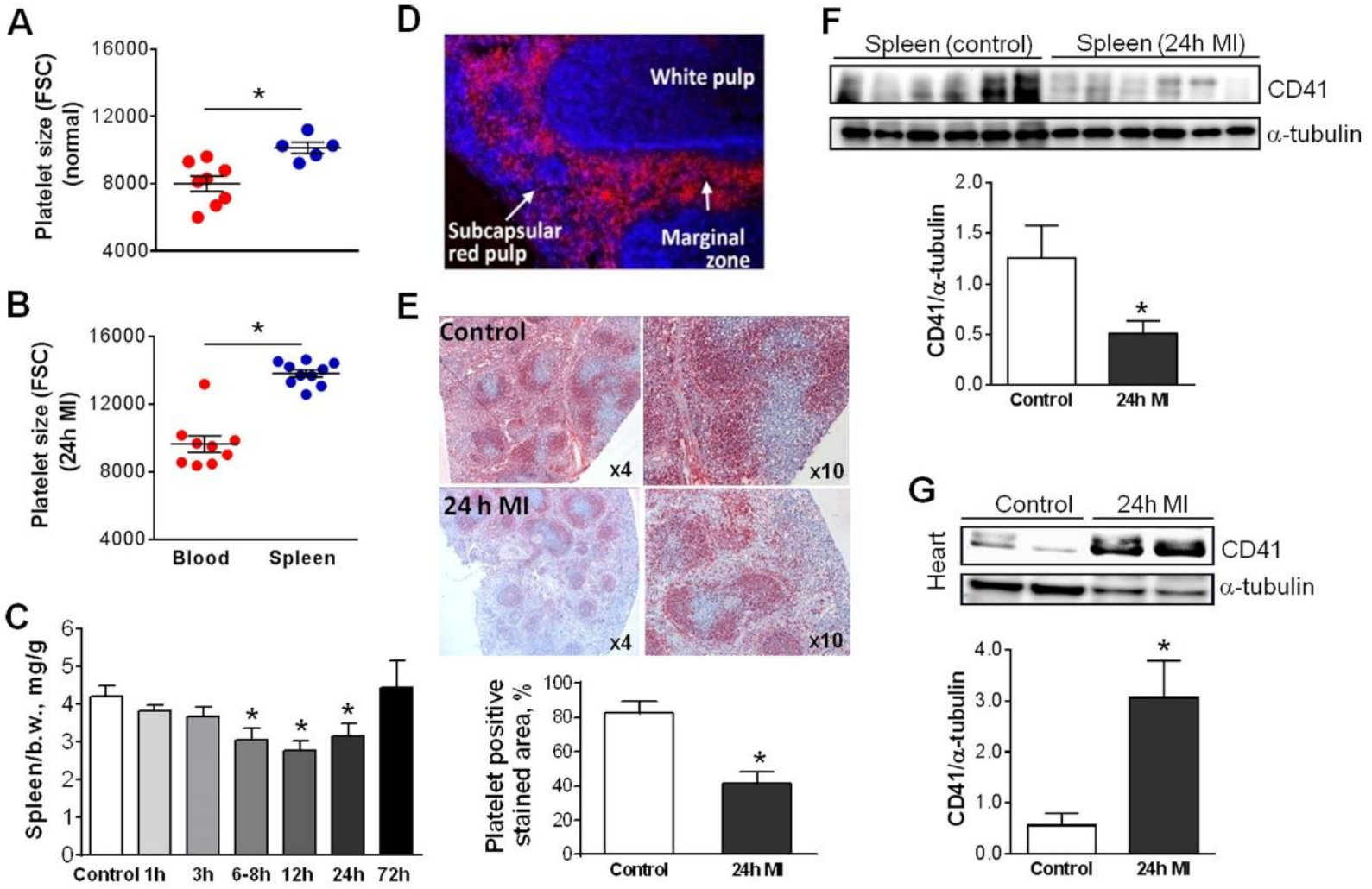
A
Platelet size (FSC) (normal)
16000
12000
8000
4000
*
B
Platelet size (FSC) (24h MI)
16000
12000
8000
4000
*
Blood
Spleen
C
Spleen/b.w., mg/g
6
5
4
3
2
1
0
*
*
*
Control 1h
3h
6-8h
12h
24h
72h
D
White pulp
Subcapsular red pulp
Marginal zone
E
Control
x4
x10
24 h MI
x4
x10
Platelet positive stained area, %
100
80
60
40
20
0
*
Control
24h MI
F
Spleen (control)
Spleen (24h MI)
CD41
α-tubulin
CD41/α-tubulin
2.0
1.5
1.0
0.5
0.0
*
Control
24h MI
G
Control
24h MI
Heart
CD41
α-tubulin
CD41/α-tubulin
4.0
3.0
2.0
1.0
0.0
*
Control
24h MI

Figure 2

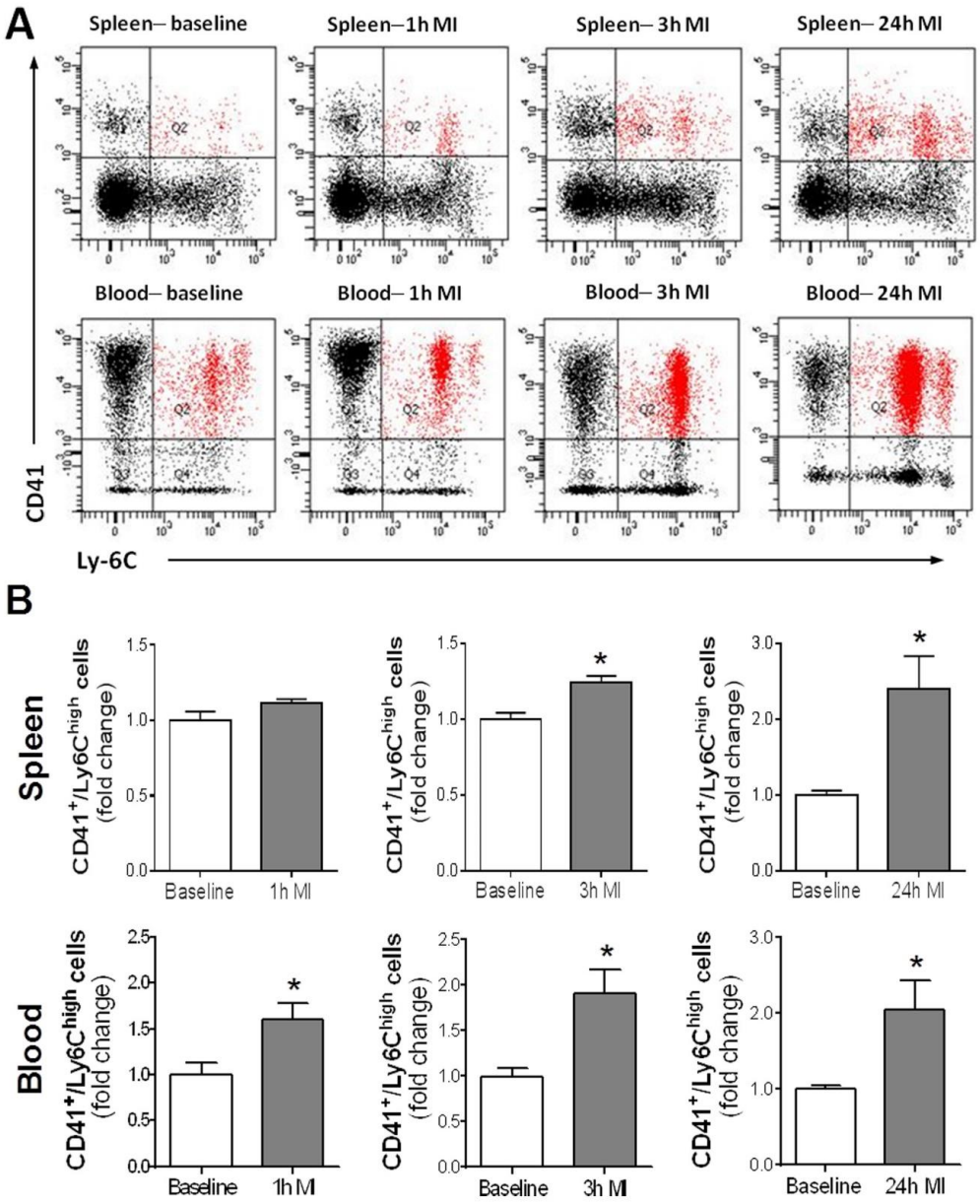
A
Spleen– baseline
Spleen–1h MI
Spleen– 3h MI
Spleen– 24h MI
Blood– baseline
Blood– 1h MI
Blood– 3h MI
Blood– 24h MI
CD41
Ly-6C
B
Spleen
Blood
CD41+/Ly6Chigh cells (fold change)
Baseline
1h MI
3h MI
24h MI

Figure 3

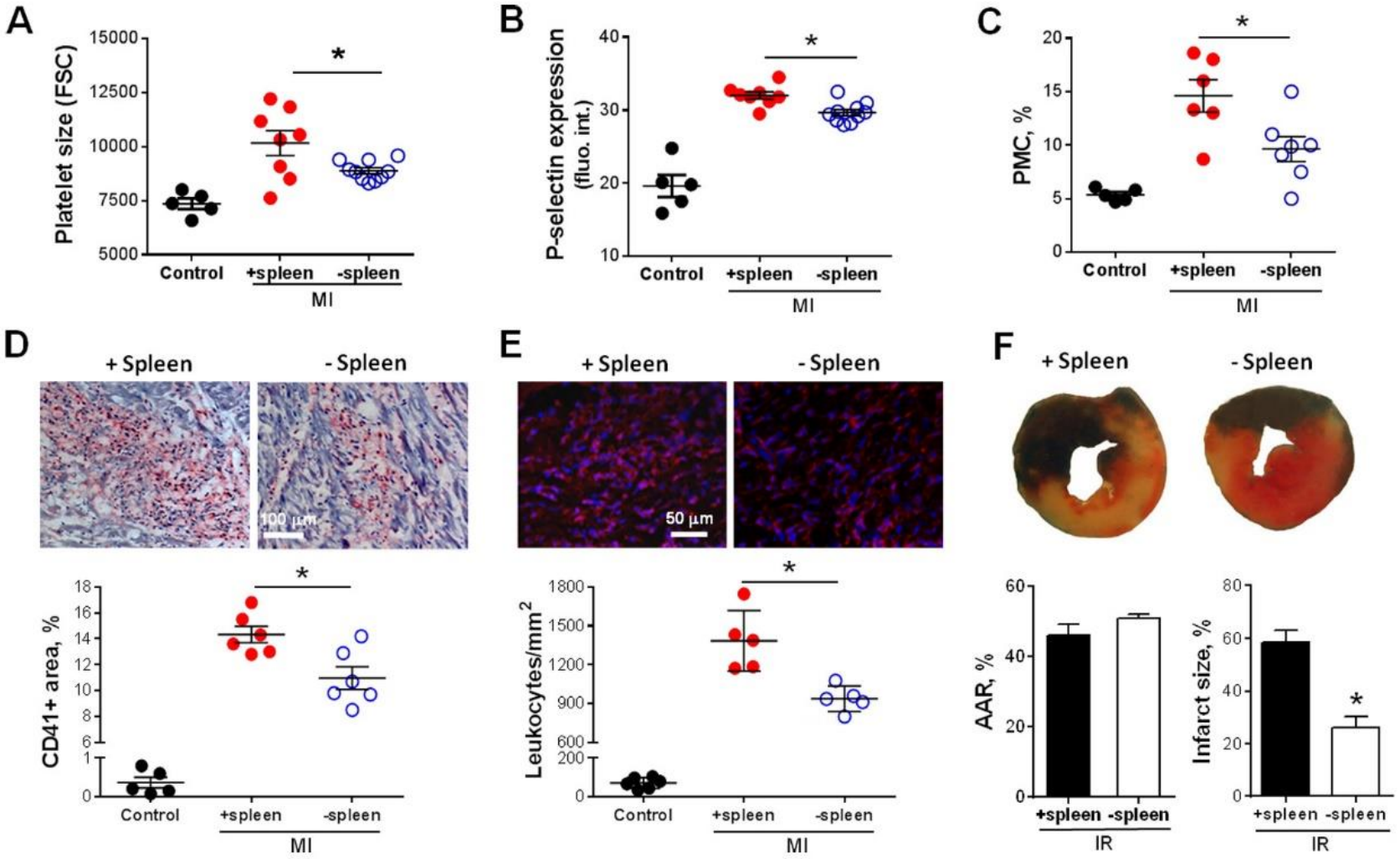
A
Platelet size (FSC)
15000
12500
10000
7500
5000
*
Control
+spleen
-spleen
MI
B
P-selectin expression
(fluo. int.)
40
30
20
10
*
Control
+spleen
-spleen
MI
C
PMC, %
20
15
10
5
0
*
Control
+spleen
-spleen
MI
D
+ Spleen
- Spleen
100 µm
CD41+ area, %
18
16
14
12
10
8
6
1
0
*
Control
+spleen
-spleen
MI
E
+ Spleen
- Spleen
50 µm
Leukocytes/mm2
1800
1500
1200
900
600
200
0
*
Control
+spleen
-spleen
MI
F
+ Spleen
- Spleen
AAR, %
60
40
20
0
+spleen
-spleen
IR
Infarct size, %
80
60
40
20
0
*
+spleen
-spleen
IR

Figure 4

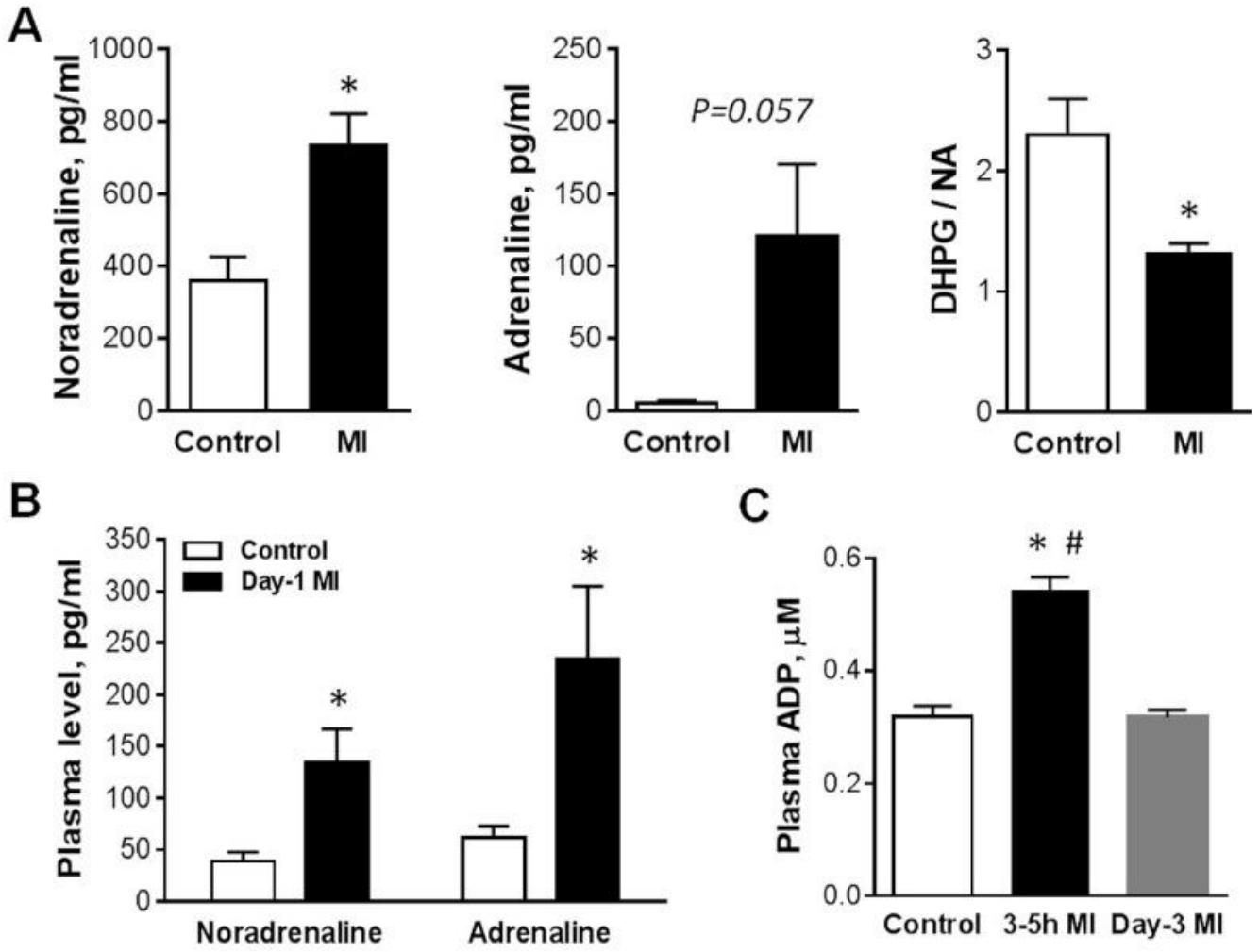
A
Noradrenaline, pg/ml
1000
800
600
400
200
0
*
Control
MI
Adrenaline, pg/ml
250
200
150
100
50
0
P=0.057
Control
MI
DHPG / NA
3
2
1
0
*
Control
MI
B
Plasma level, pg/ml
350
300
250
200
150
100
50
0
Control
Day-1 MI
*
*
Noradrenaline
Adrenaline
C
Plasma ADP, µM
0.6
0.4
0.2
0.0
* #
Control
3-5h MI
Day-3 MI

Figure 5

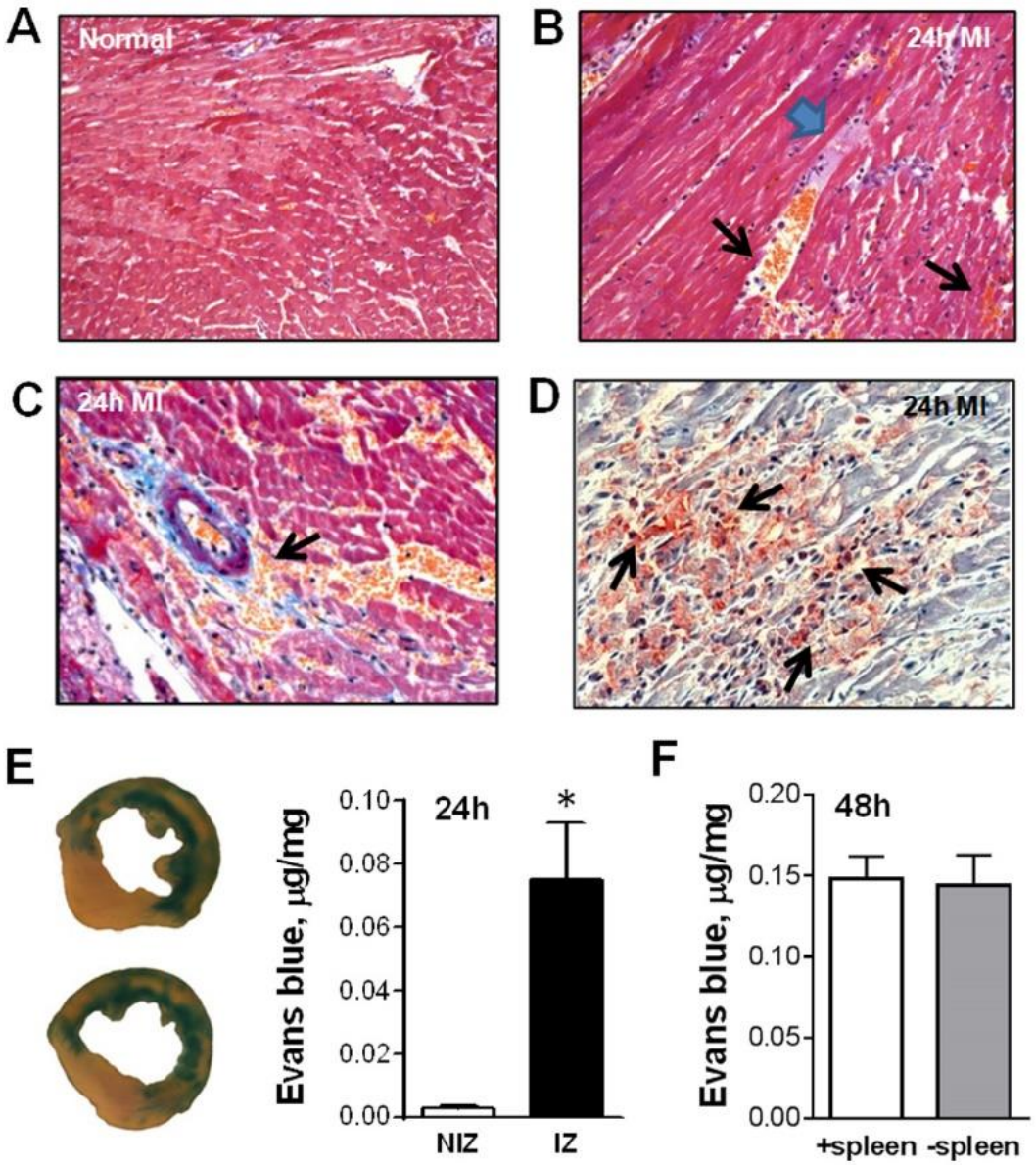
A
Normal
B
24h MI
C
24h MI
D
24h MI
E
Evans blue, µg/mg
0.10
0.08
0.06
0.04
0.02
0.00
24h
*
NIZ
IZ
F
Evans blue, µg/mg
0.20
0.15
0.10
0.05
0.00
48h
+spleen
-spleen

Figure 6

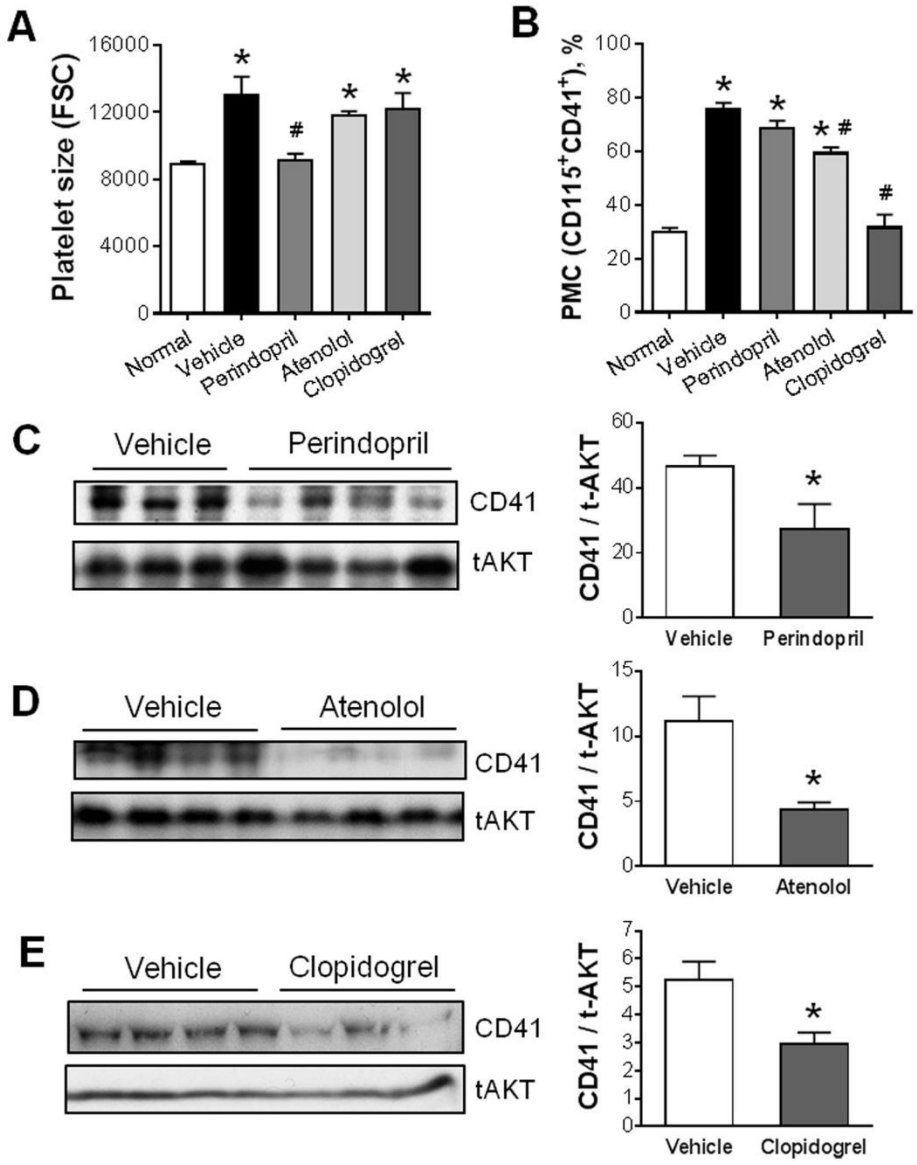
A
Platelet size (FSC)
16000
12000
8000
4000
0
Normal
Vehicle
Perindopril
Atenolol
Clopidogrel
B
PMC (CD115+CD41+), %
100
80
60
40
20
0
C
Vehicle
Perindopril
CD41
tAKT
CD41 / t-AKT
60
40
20
0
D
Vehicle
Atenolol
15
10
5
E
Vehicle
Clopidogrel
7
6
5
4
3
2
1

Figure 7

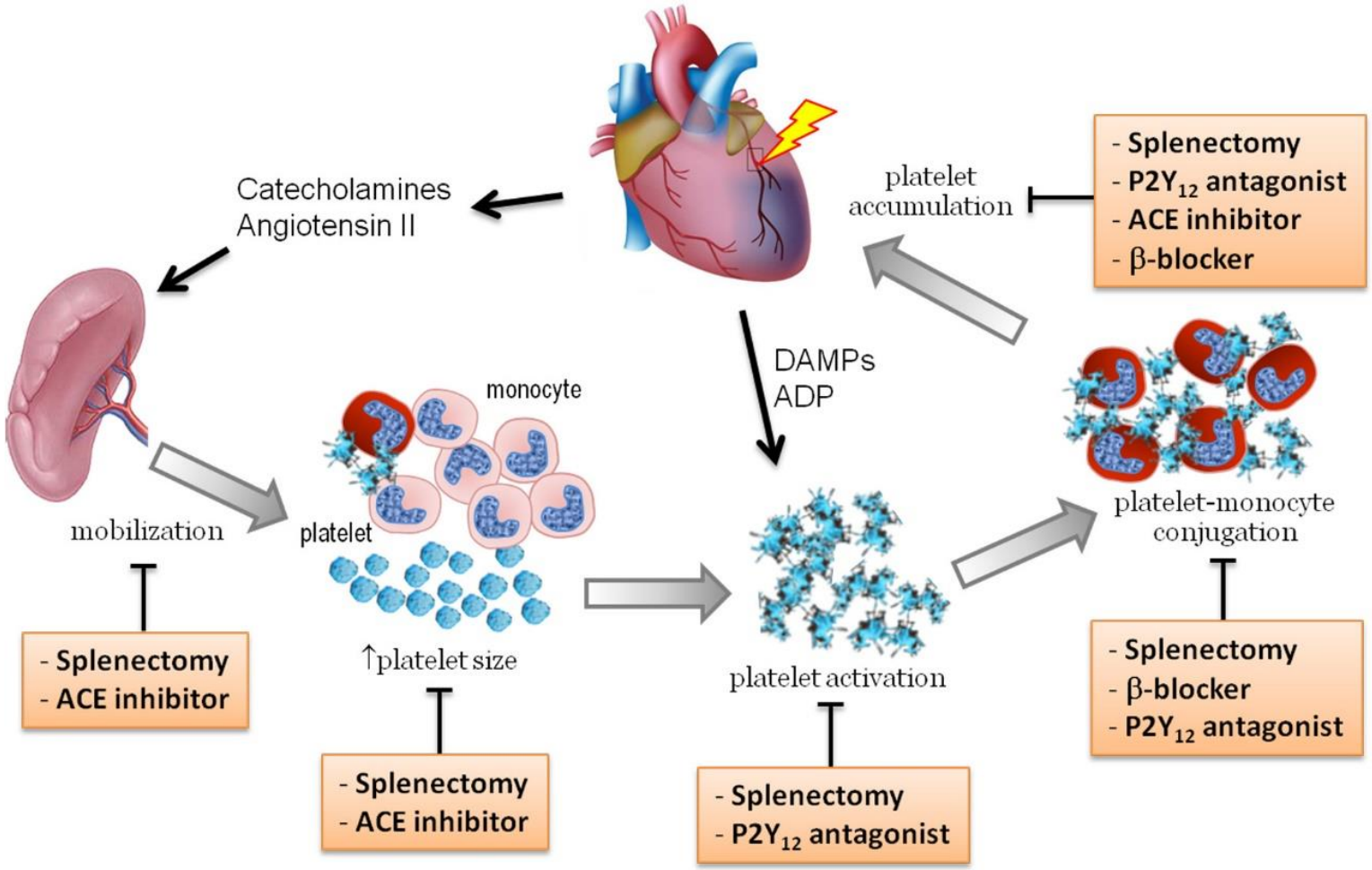
Catecholamines
Angiotensin II
platelet accumulation
- Splenectomy
- P2Y12 antagonist
- ACE inhibitor
- β-blocker
DAMPs
ADP
monocyte
platelet
mobilization
- Splenectomy
- ACE inhibitor
↑platelet size
- Splenectomy
- ACE inhibitor
platelet activation
- Splenectomy
- P2Y12 antagonist
platelet-monocyte conjugation
- Splenectomy
- β-blocker
- P2Y12 antagonist

Figure 8