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

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

*Experimental Cardiology Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, VIC 3004, Australia
†Central Clinical School, Monash University, Melbourne, VIC 3004, Australia
‡Alfred Heart Centre, Alfred Hospital, Melbourne, VIC 3004, Australia
§Department of Cardiology, Third Hospital of Peking University, Beijing 100191, China

Abstract

Acute myocardial infarction (AMI) is characterized by a rapid increase in circulating platelet size but the mechanism for this is unclear. Large platelets are hyperactive and associated with adverse clinical outcomes. We determined mean platelet volume (MPV) and platelet–monocyte conjugation (PMC) using blood samples from patients, and blood and the spleen from mice with AMI. We further measured changes in platelet size, PMC, cardiac and splenic contents of platelets and leucocyte infiltration into the mouse heart. In AMI patients, circulating MPV and PMC increased at 1–3 h post-MI and MPV returned to reference levels within 24 h after admission. In mice with MI, increases in platelet size and PMC became evident within 12 h and were sustained up to 72 h. Splenic platelets are bigger than circulating platelets in normal or infarct mice. At 24 h post-MI, splenic platelet storage was halved whereas cardiac platelets increased by 4-fold. Splenectomy attenuated all changes observed in the blood, reduced leucocyte and platelet accumulation in the infarct myocardium, limited infarct size and alleviated cardiac dilatation and dysfunction. AMI-induced elevated circulating levels of adenosine diphosphate and catecholamines in both human and the mouse, which may trigger splenic platelet release. Pharmacological inhibition of angiotensin-converting enzyme, β_1-adrenergic receptor or platelet P2Y$_{12}$ receptor reduced platelet abundance in the murine infarct myocardium albeit having diverse effects on platelet size and PMC. In conclusion, AMI evokes release of splenic platelets, which contributes to the increase in platelet size and PMC and facilitates myocardial accumulation of platelets and leucocytes, thereby promoting post-infarct inflammation.

Key words: catecholamines, infarct size, inflammation, mean platelet volume, monocytes, myocardial infarction, platelets, spleen.

INTRODUCTION

In the pathophysiology of myocardial infarction (MI), platelets exert important 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 MI (AMI) [8], and a pivotal role of circulating platelet–monocyte conjugation (PMC) contributing to inflammation and platelet accumulation in the infarct mouse heart [5,9].

Patients with AMI manifest an increase in circulating platelet size, measured as mean platelet volume (MPV) [10–18]. Large MPV is associated with adverse cardiovascular events and poor survival [10,14,17–19]. In addition, MPV returns to reference range within the first day after commence of therapies [18].

These findings raise some key questions: What is the mechanism responsible for such prompt increase in platelet size? Whether large platelets are important in the setting of AMI? Whether current medications are responsible for resolution in platelet size and activity soon afterwards?

The mechanism for the enlarged circulating platelets at the time of admission, however, remains unclear. Newly generated platelets by megakaryocytes are usually bigger in size [20,21]. 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 [20–22]. Thus, it is less likely that bone marrow release of newly generated bigger platelets is responsible for the rapid increase in MPV following AMI.

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 [23–26]. Hence, the term 'cardiosplenic axis' is used to highlight the splenic contribution to post-MI innate immune response [23,24,27]. The spleen is also known to store (approximately 30 % of total platelets), sequester and release platelets. Further, relative to circulating platelets, splenic platelets are 20–30 % bigger in size and mobilization of splenic platelets could enlarge circulating MPV as previously shown under stressed conditions including intense exercise and stimulation by cytokines or catecholamines [28–31].

We hypothesize that AMI 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 AMI to assess platelet-related parameters in the blood, spleen and myocardium. Influences of splenectomy and drugs routinely used in patients with AMI on these measures were also studied in mice subjected to MI.

METHODS

Recruitment of human patients with AMI

To explore temporal changes in circulating platelet size and PMC in patients with AMI, 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 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 from patients with MI at the time of admission (average 3 h after onset of MI) prior to PCI or medication, and then at 24 and 72 h after admission, and from healthy volunteers or age- and gender-matched CAD patients using 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, 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°C for 30 min in the dark, washed with PBS and then fixed in 2 % paraformaldehyde. FACS was performed on BD FACSCalibur^{TM} using appropriate settings excluding debris. Twenty thousand 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 anaesthetized 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 incision was closed by stitches in layers. To determine the influence of splenectomy on the infarct size, some mice without and with splenectomy 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 thoracotomy 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 anaesthetized using isoflurane at 4.5 % for induction and 1.7 % for maintenance. Transthoracic echocardiography was performed using a Vevo 2100 ultrasound system (VisualSonics) 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 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 impact of MI, animals with an infarct size less than 30% or over 55% of the LV were excluded. Tissues collected were either freshly processed or stored at −80°C till subsequent use. In experiments with splenectomy, infarct size was also determined at 24 h after ischaemia–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, 24 and 72 h post-MI, together with control mice, blood samples (⩾20 μl) collected from tail veins with Microvette® 200 containing EDTA were analysed using Hemavet 950FS Blood Analyser (Drew Scientific) for platelet counting according to the manufacture's instruction.

Flow cytometry in animal studies

Platelet size and PMC

To assess post-MI changes in platelet size and PMC, mouse blood was collected at 12, 24 and 72 h post-MI by cardiac puncture with heparin as anticoagulant. Platelet-rich plasma was prepared and 10000 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 20000 events per sample were collected within a monocyte gate by BD FACSCalibur™, and analysed using the FlowJo software (Tree Star). 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 of 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 infarcted mice with or without splenectomy. 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} leucocytes in mouse blood and spleen

To understand the role of platelets in post-MI inflammation, we measured changes of platelet conjugation with Ly-6C^{high} inflammatory leucocytes, in response to MI, both in the spleen and 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 (300 g for 8 min). Leucocytes were isolated from 50 μl blood following lysis of red blood cells and centrifugation (300 g for 8 min). They were resuspended in the FACS buffer (100 μl). Both splenic cells and blood leucocytes 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™. Fifty thousand 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.

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 post-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. DAPI was applied to stain nuclei. Ten histological images were acquired per tissue sample and CD41-positive stained regions were analysed digitally using ImagePro software and expressed as the percentage of imaging area [5].

Quantification of leucocyte density

Mouse heart frozen sections collected at 48 h post-MI were stained with rat-anti-mouse CD45 antibody (BD Biosciences), 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 leucocytes were counted manually and expressed as average cell number per mm^2, as we previously described [5].

Co-localization of platelets and leucocytes within the infarct myocardium

Freshly frozen hearts at 48 h post-MI were used. Similar to 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 CD41 antibody (BD Biosciences), as we previously described [5]. Results were expressed as ratio of house-keeping proteins (α-tubulin or total-AKT).

Assays for ADP or catecholamines in plasma

ADP levels in the plasma samples from patients with STEMI were determined using ADP-Glo^{TM} Kinase Assay kit (Promega) following the supplier's instructions. This assay involves converting ADP into ATP by a kinase reaction and then into light by Ultra-Glo^{TM} Luciferase. Care was taken to prevent haemolysis of blood samples. Adrenaline and noradrenaline 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 leakage following MI

MI is associated with microvascular damage which could facilitate extravascular platelet accumulation and inflammatory cell infiltration. 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, the animal was anaesthetized. 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}). 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 Laboratories), calculated against standard curve and normalized by tissue weight.

Effects of angiotensin-converting enzyme inhibitor (ACEI), β_1-adrenergic receptor (β_1-AR) blocker and platelet P2Y$_{12}$ receptor antagonist

To explore the mechanism of splenic platelets release following MI, we treated MI mice with, the angiotensin-converting enzyme inhibitor (ACEI) perindopri (Servier, 6 mg/kg), β_1-AR blocker atenolol (Sigma, 2.5 mg/kg) or vehicle (saline) respectively. The drugs were given i.p. in a bolus immediately before coronary artery occlusion followed by addition in drinking water. Platelet P2Y$_{12}$ receptor antagonist clopidogrel (Lilly, 50 mg/kg) was given by gavage 1 h after induction of MI [5]. Blood and heart tissues were collected 24 h post-MI for measurement of platelet size (FSC) and PMC (% of CD115$^+$CD41$^+$ PMC over CD115$^+$ monocytes) by FACS and platelet abundance in the infarct myocardium by immunoblotting using anti-CD41 antibody.

Statistics

Results are expressed as mean $\pm$ S.E.M., unless otherwise stated. Using GraphPad Prism software, data were analysed by ANOVA, 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 with that of healthy controls or patients with stable CAD. Table 1 shows basic clinical data of the three groups. Compared with the healthy control group, patients with STEMI were older, had increased body mass index, higher incidences of hypertension, diabetes and hyperlipidaemia and were more likely to smoke (Table 1). Moreover, our STEMI patient cohort had much lower rates of pre-use of β-blocker, ACEI or angiotensin II receptor blocker (ARB), aspirin and statins than stable CAD patients although the pre-use of aspirin was 89% (Table 1). The 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 compared with the other two groups. MPV, however, was significantly greater in STEMI compared with the two control groups (Table 1). To trace time-dependent change in MPV and platelet count, STEMI patients were re-grouped, based on symptom-to-blood sampling time, into subgroups ($n = 38$–80 per subgroup). STEMI patients showed a prompt increase in MPV as early as 1 h after onset of MI by approximately 20% over control values ($P < 0.01$). MPV then returned to the reference level within 24 h after commencement of routine treatment and remained at this level at 72 h post-MI (Figure 1A). Platelet count did not change (Figure 1B). In STEMI patients, PMC was increased by approximately 3-fold ($P < 0.05$) at the time of admission and then declined when measured at 24 and 72 h (Figure 1C).

Mice subjected to MI showed a significant elevation in platelet size and PMC as early as 12 h post-MI (Figures 1D and 1E), although platelet count was comparable to control mice during this period (Figure 1F). In contrast with findings in patients, changes in platelet size and PMC were sustained at 72 h post-MI in infarcted mice.

Platelet size comparison and reduction in the splenic platelet store post-MI

We first compared the platelets size isolated from blood and the spleen in mice without and with MI. Splenic platelets were approximately 25% larger than circulating platelets under normal conditions ($P < 0.01$, Figure 2A). At 24 h post-MI, the size of circulating and splenic platelets increased with splenic platelets remaining bigger by 46% than circulating platelets ($P < 0.01$, Figure 2B).

Table 1   Basic clinical and haematological parameters in healthy subjects and patients with stable CAD or AMI
Data are mean $\pm$ S.D. *$P < 0.05$ compared with healthy control, $^\dagger P < 0.05$ compared with stable CAD.

	Healthy control	Stable CAD	STEMI
Number	55	25	344
Male gender (%)	65	52	80†
Age (year)	54 $\pm$ 10	62 $\pm$ 9*	61 $\pm$ 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 $\pm$ 2.5	25.9 $\pm$ 3.6*	24.0 $\pm$ 2.4*
Platelet count (10^9/L)	203 $\pm$ 41	222 $\pm$ 66	221 $\pm$ 59
Mean platelet volume (fL)	8.6 $\pm$ 0.9	8.4 $\pm$ 1.1	10.1 $\pm$ 1.0*†
Drug pre-use			
β-blocker	0	84%*	6.1%†
ACEI or ARB	0	72%*	9.9%†
Aspirin	0	92%*	89%*
Statins	0	88%*	5.8%†

The spleen underwent a transient reduction in its mass during the period of 6–24 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. The CD41$^+$ stained region shrank by approximately 50% at 24 h after MI ($P < 0.05$) compared with 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-fold increase in CD41 expression in the infarct myocardium ($P < 0.05$, Figure 2G).

Circulating and splenic platelet-inflammatory leucocyte 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 1.5–2-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 approximately 20% at 3 h ($P < 0.05$) and a further to 2.4-fold at 24 h over the baseline value (Figures 3A and 3B).

Effects of splenectomy on platelet and inflammatory parameters, infarct size and ventricular remodelling

Splenectomy was applied to confirm the role of the spleen in the changes observed following MI. Blood was collected at 24 h and hearts collected at 48 h post-MI in mice without and with splenectomy and normal mice served as the control. Although MI induced a significant increase in all inflammatory parameters of blood (24 h) and the infarct myocardium (48 h) (Figures 4A–

4E), 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 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 leucocyte 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 with that of intact animals and a trend for a reduced infarct size (Table 2). In splenectomized mice subjected to ischaemia–reperfusion, the infarct size was 55% smaller compared with intact mice ($P < 0.001$, Figure 4F), whereas area at risk (AAR) was comparable (Figure 4F).

Elevated circulating levels of catecholamines and ADP following AMI

Plasma was prepared from admission blood samples in STEMI patients. Concentrations of noradrenaline were increased by 2-fold ($P < 0.05$, Figure 5A) and that of adrenaline tended to be higher than control values ($P = 0.057$). The ratio of dihydroxyphenylglycol (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 AMI, 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 ischaemia induced microvascular leakage

At 24 h post-MI, intramyocardial haemorrhage was detected within the infarct region by Carstair's staining (Figures 6B
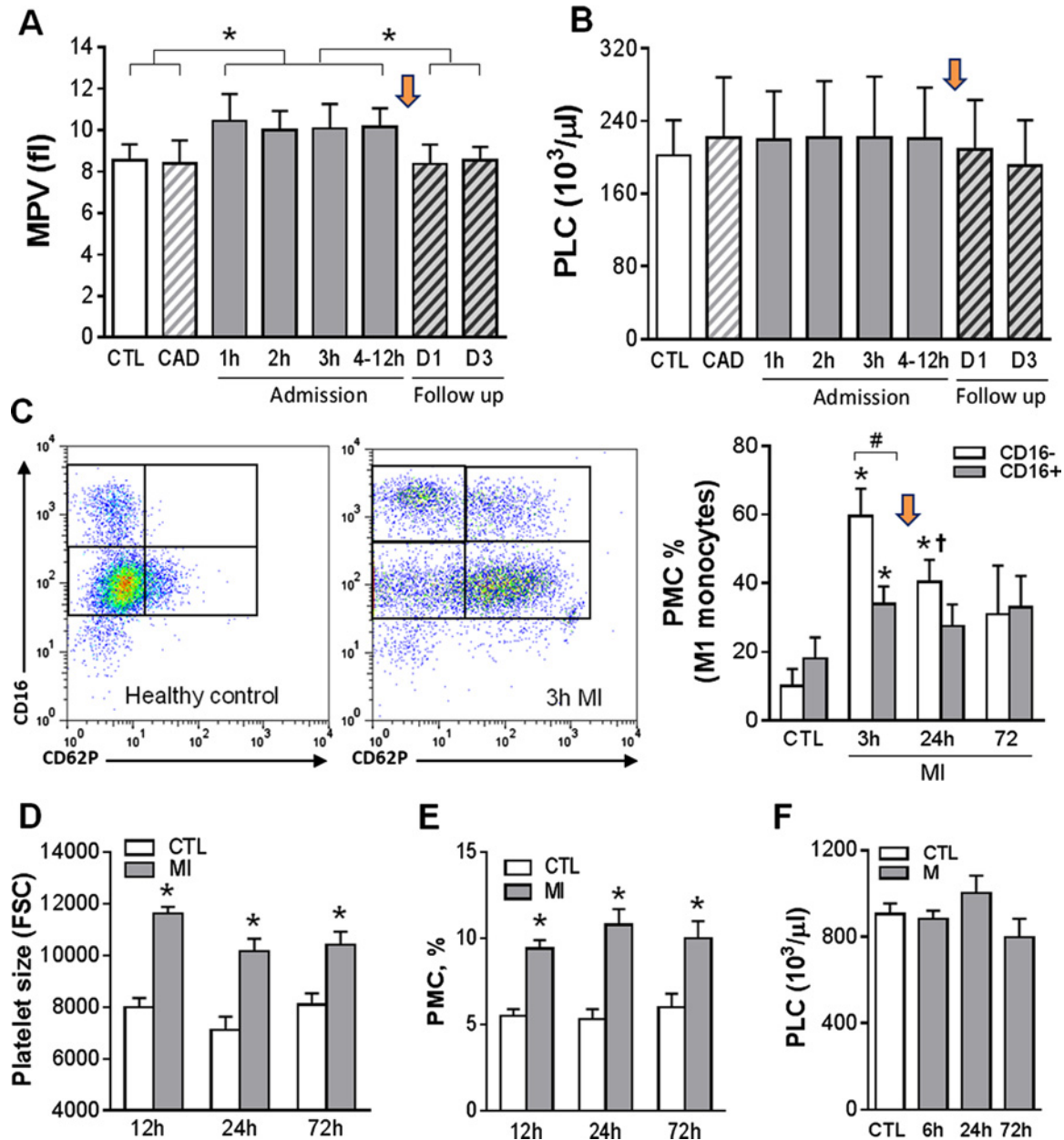
**Figure 1 Dynamic changes in platelet size and PMC in human patients or mice following MI**

(**A** and **B**) Changes in human patients of MPV (**A**) and platelet count (PLC, **B**) at the time of admission and during follow-up. For admission data, patients ($n = 344$) were re-grouped ($n = 38–133$) according the symptom-to-blood sampling time. Arrows indicate commencement of therapies. Results from healthy subjects ($n = 55$) and age- and gender-matched patients with CAD ($n = 25$) are presented as reference. Results are mean $\pm$ S.D. *$P < 0.01$. (**C**) Representative FACS plots and grouped data showing increased PMC in patients with AMI ($n = 7$/group) with results presented as PMC percentage of total CD16$^-$ cells (M1 monocytes). Results are mean $\pm$ S.D. *$P < 0.05$ compared with the same cell type in control (CTL), †$P < 0.05$ compared with 3 h CD16$^-$. (**D** and **F**) Changes of platelet size (**D**), PMC (**E**) and platelet count (**F**) in mice at different time points after coronary artery occlusion. Results are mean $\pm$ S.E.M., $n = 7–9$/group. *$P < 0.05$ compared with CTL.

and 6C). Immunohistochemistry showed a close co-localization of platelets (CD41$^+$ staining in red) and infiltrated leucocytes cells (CD45$^+$ nuclei staining in dark) 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 with non-infarct tissues (Figure 6E) 24 h post-MI, suggesting microvascular leakage that could facilitate myocardial infiltration by circulating platelets and leucocytes. Splenectomy had no effect on Evans Blue content in the infarct myocardium at 48 h post-MI when microvascular leakiness is more severe (Figure 6F).

Table 2   Influence of splenectomy in LV remodelling and function at 48 h after MI
Data are mean $\pm$ S.E.M. *$P<0.01$ compared with sham, [†]$P < 0.01$ compared with MI, intact group.

|	Heart rate (beats/min)	LV area-systole (mm²)	LV area-diastole (mm²)	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
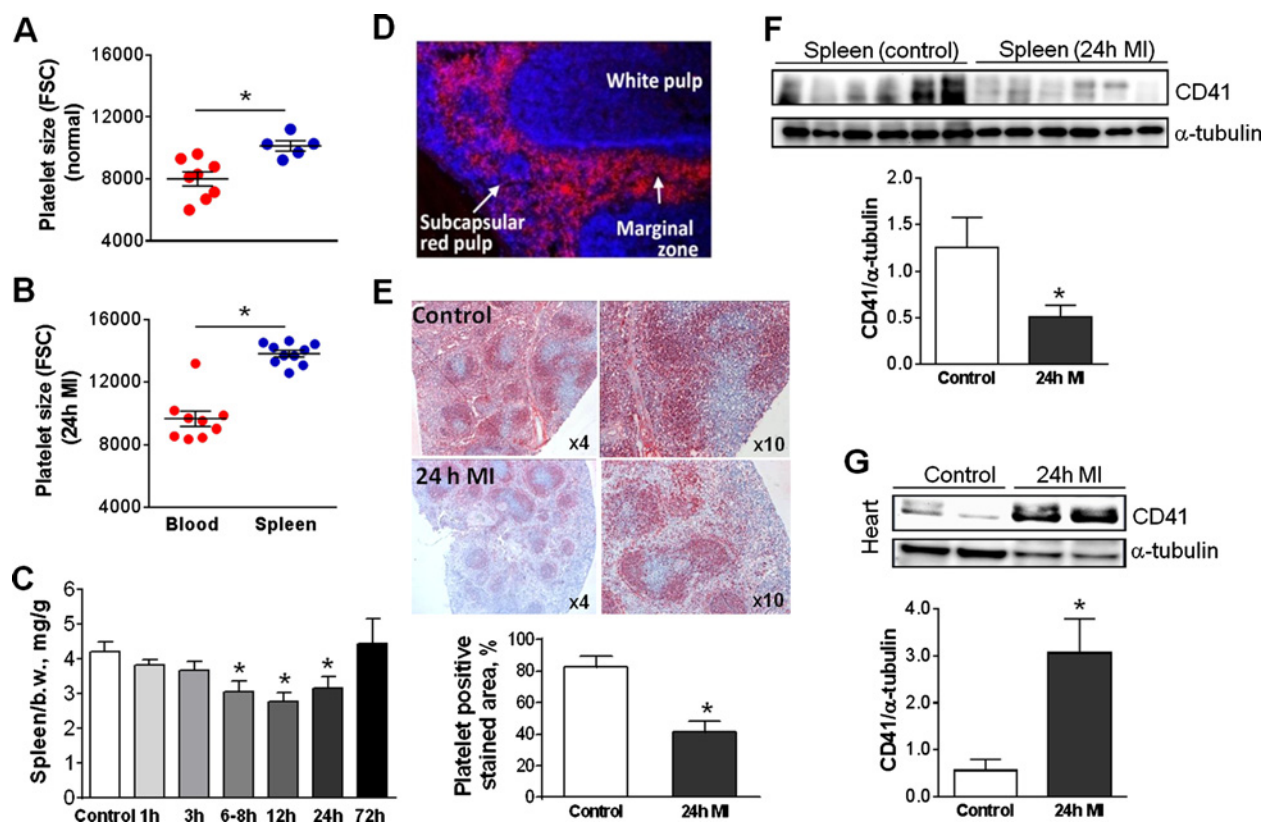
Figure 2   Changes in platelet size, spleen weight and tissue platelet content in mice after MI
(**A** and **B**) Splenic platelets were larger than blood platelets in normal mice (**A**) and mice at 24 h post-MI (**B**). *$P<0.01$. (**C**) Transient reduction in the spleen weight normalized by body weight during 6–72 h after MI. $n = 8$–12 per MI group and $n = 18$ for control group. (**D**) Immunofluorescence (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 compared with sham-operated mice. (**F**) Reduction in platelet content determined by CD41 abundance by immunoblotting in the spleen 24 h after MI compared with control mice ($n = 6$/group). (**G**) Increase in CD41 expression by immunoblotting of the infarct myocardium at 24 h post-MI ($n = 5$–6/group). *$P < 0.05$ compared with control values (C–G).

Distinct effects of ACEI, β_1-blocker and platelet P2Y$_{12}$ antagonist on platelet parameters in mice with AMI

Following the observation of differential dynamic changes in platelet size and MPV between human patients and mice post-MI (Figure 1), we tested effects of drugs routinely used in patients with AMI on platelet parameters. Mice were treated with perindopril, atenolol or clopidogrel, and changes in platelet size and PMC in circulating blood as well as CD41 abund-ance in the infarct myocardium were measured at 24 h after MI. Compared with vehicle-treated mice, perindopril largely abolished the increase in platelet size but the other two drugs had no such effect ($P < 0.05$, Figure 7A). In contrast, PMC was reduced by 22% by atenolol and abolished by clopido-grel, but unaffected by perindopril (Figure 7B). Importantly, all three drugs tested similarly reduced myocardial accumu-lation of platelets by 40–60% at 24 h post-MI (all $P < 0.05$, Figures 7C–7E).
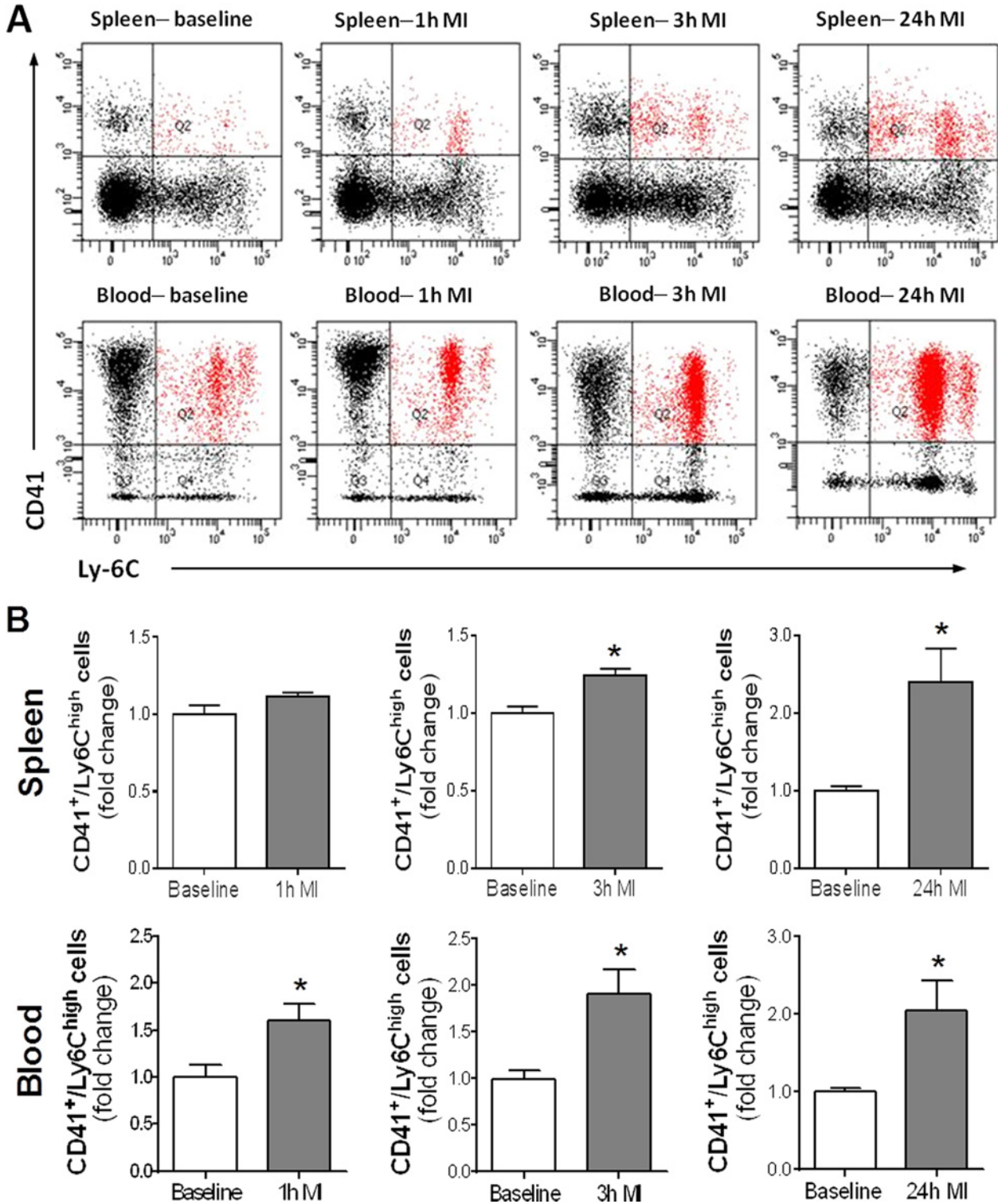
Figure 3 Temporal changes of PMC in the spleen and blood in mice after MI
(**A**) Representative FACS plots displaying changes in platelet and inflammatory monocyte conjugation determined by the population of cells expressing high levels of CD41 and Ly-6C in the spleen and blood samples from mice at baseline or at 1, 3 and 24 h post-MI. Quantitative data are presented in panel (**B**), $n = 5–7$/group. *$P < 0.05$ compared with baseline values.
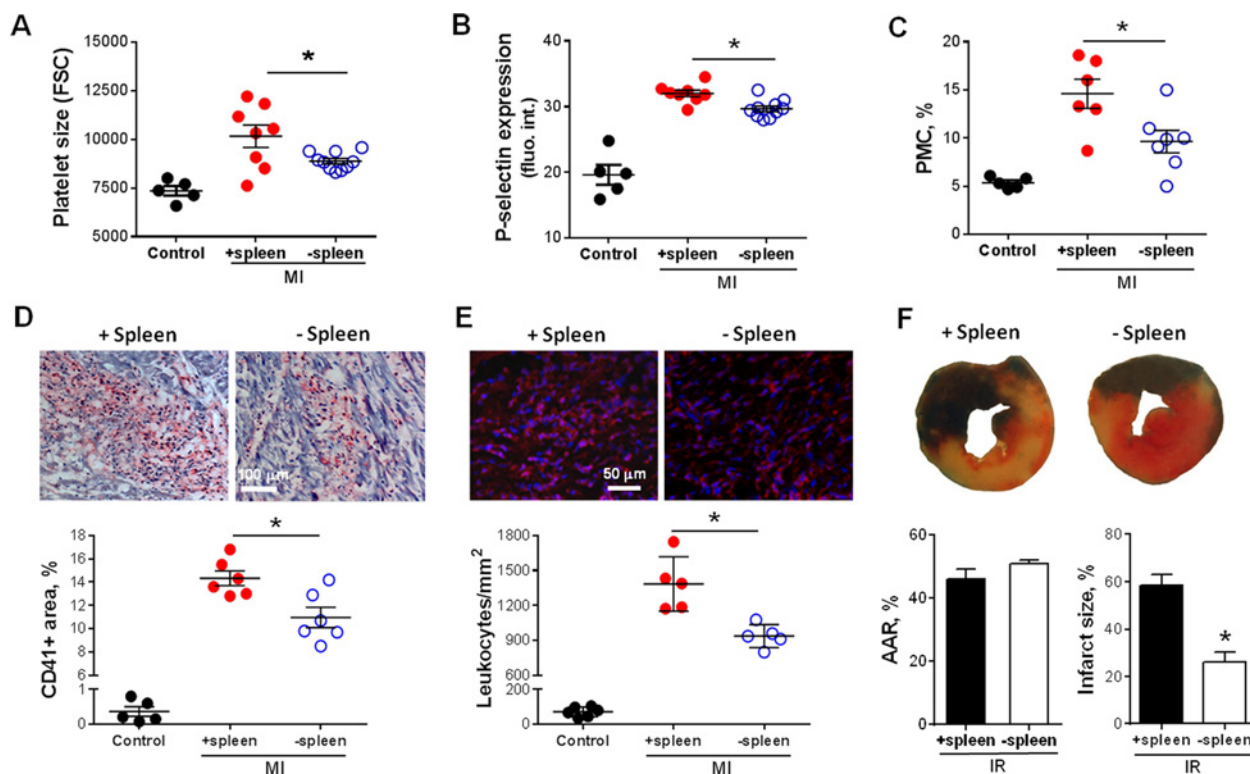
Figure 4   Effects of splenectomy on platelet and inflammatory parameters and the infarct size in mice with AMI
(**A**–**C**) Compared with intact mice, splenectomy significantly reduced circulating platelet size (**A**), platelet activation measured by CD62P^{+} platelets (P-selectin, **B**) and PMC measured as CD45^{+}/CD41^{+} monocytes over total monocytes analysed by FACS (**C**) at 24 h post-MI. *$P < 0.05$. (**D** and **E**) In the infarct myocardium, splenectomy notably reduced platelet accumulation (CD41^{+} stained area, red colour, **D**) and leucocyte infiltration (CD45^{+} stained cells showing pink with blue DAPI stained nuclei, **E**) at 48 h post-MI compared with intact mice. *$P < 0.05$. (**F**) Splenectomy reduced infarct size at 24 h following ischaemia–reperfusion (IR) despite a comparable AAR between groups with or without splenectomy. $n =$ 9–10/group. *$P < 0.001$ compared with intact mice (+spleen).

DISCUSSION

Our study provides several novel findings. First, we document splenic platelet release as a mechanism responsible for the rapid increase in circulating MPV shortly after MI. Second, 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 leucocytes. Third, splenectomy attenuated the sequential changes of systemic and regional inflammation and consequently, reduced infarct size and alleviated ventricular remodelling and dysfunction at the acute phase following MI. Fourth, inhibitors targeting β_1-AR, ACE or P2Y$_{12}$ attenuated MI-induced changes in platelet parameters including platelet accumulation in the infarct myocardium. Thus, the spleen acts as a key organ in the systemic response to acute ischaemic injury by releasing monocytes and platelets to promote inflammation, and splenic platelets are another player in the 'cardiosplenic axis' (Figure 8).

Platelet size is an indirect index and enlarged platelet size is associated with increased platelet activity [36]. In the setting of AMI, large MPV is associated with increased risk for acute stent thrombosis [13], no-reflow post PCI [12,14,16–18], re-infarction

[17,37], larger infarct size [12], pump failure [18,38], blunted response to anti-platelet drugs [15,39] and short- and long-term mortality [13,17,37,40]. Numerous clinical studies have reported increase in MPV at day-1 after AMI [10,12–15]. In the present study, such change occurs in STEMI patients as early as 1 h following symptom onset. Circulating platelets are heterogeneous in size, density and reactivity [20–22]. Large platelets are in general hyperactive in functionality, such as aggregation in response to collagen or ADP, thromboxane release and membrane expression of P-selectin or GP1b, GPIIb/IIIa [19–21]. Large platelets are also high in granule content and hence granular release of active molecules including ADP and serotonin, and yet resistant to anti-platelet drugs [13,15,18,19,36,41]. Platelets have been implicated in mediating cardiac damage and remodelling, measured as regional inflammation, wall rupture, intramyocardial thrombus, which were attenuated by experimental thrombocytopenia or P2Y$_{12}$ inhibitors in infarct mice [5,9]. In further support of the beneficial effects of platelet inhibition reported by us, we showed in the present study that splenectomy also attenuated inflammatory infiltration and limited the infarct size.

Although newly generated platelets are usually large in size [20–22], at least a 24-h period is required for *de novo* generation and release of platelets by mature megakaryocytes upon
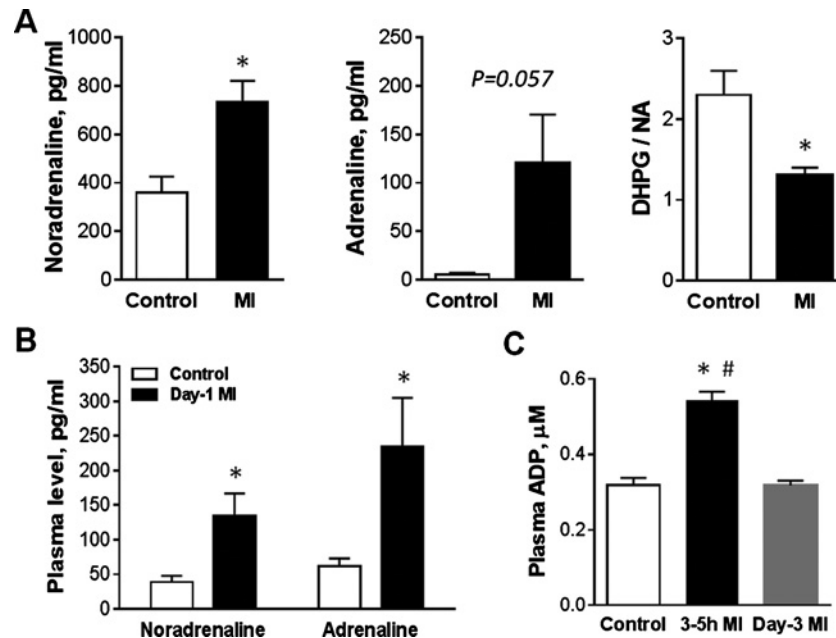
Figure 5   Increased plasma levels of catecholamines and ADP in patients or catecholamines in mice with MI

(**A**) Elevated plasma levels of noradrenaline (NA) and adrenaline, and decreased neuronal reuptake activity indicated by the lower ratio of DHPG/NA in patients at day-1 after MI ($n = 14$) in comparison with 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 AMI at the time of admission (3–5 h post-MI) but not at day-3. $*P < 0.05$ compared with control, $\#P < 0.05$ compared with day-3 MI values.

stimuli such as cytokines [20,42]. 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 rapid increase in platelet size is due to splenic release of stored platelets, we studied in mice before and after MI temporal changes of platelet number and size both in blood and the spleen and examined effects of splenectomy. Splenic platelets in normal mice were 25% bigger than blood platelets, and platelet size increased further in blood and the spleen following MI. Importantly, splenectomy blunted all these changes induced by MI. These data indicate that release of splenic platelets is responsible for the increase in circulating platelet size associated with MI. Release of splenic platelets post-MI was further indicated by a reduced abundance of CD41 in the spleen. Importantly, reduced splenic platelet storage following MI is accompanied by an increase in the platelet content in the infarct myocardium. 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 [5,6,43]. 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' leucocytes [3,5,7]. Swirski et al. reported that splenic release of Ly-6C^{high} monocytes is coupled with myocardial infiltration by Ly-6C^{high} monocytes [23,26]. The term 'cardiosplenic axis' is then used to highlight the contribution of the spleen to post-MI inflammatory response [23–27]. However, 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 [22,44,45]. 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 leucocytes post-MI, a dynamic process similar to that of splenic inflammatory (M1) monocytes [23,26]. Thus, splenic PMC is expected to contribute to activation and release of inflammatory monocytes into the circulation upon AMI (Figure 8).

Considering the sizable splenic storage of monocytes (>50%) [23–27] and platelets (30–40%) [22,44,45], splenectomy is expected to attenuate inflammatory response by removal of the splenic pool of Ly-6C^{high} monocytes and platelets. Indeed, in the current study, splenectomy reduced circulating platelet size, PMC and, notably, myocardial accumulation of platelets and leucocytes. Consequently, splenectomy limited the infarct size and attenuated LV dilatation and dysfunction in the acute phase. These results highlight the splenic mobilization of monocytes/platelets as a major contributor to the systemic and cardiac inflammation following ischaemic insult. These findings also extend the current understanding of pro-inflammatory properties of platelets and underscore the role of splenic platelet pool in contributing to activation of circulating leucocytes (Figure 8), particularly inflammatory monocytes [5,23,26,27,46]. Our findings support the significance of 'cardiosplenic axis' in post-MI inflammation and further demonstrate co-release of splenic platelet/monocyte as a pivotal part of such responses post-MI. As illustrated in our current study, this chain of events (Figure 8), in particular PMC as the major mechanism mediating
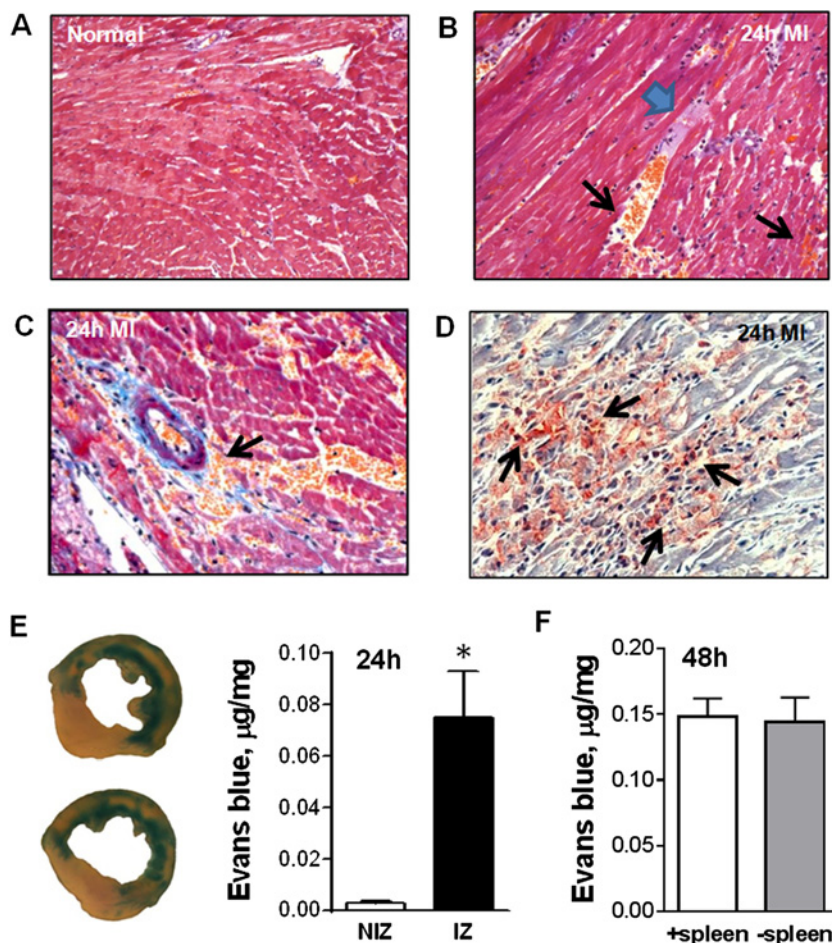
Figure 6 **Intramyocardial haemorrhage and microvascular leakiness in hearts of mice with MI**
(**A**–**C**) Carstair's staining of mouse hearts with sham-surgery (**A**) or with MI for 24 h (**B** and **C**) depicting extravascular presence of red blood cells (orange stained, black arrows) and thrombus (blue arrow). (**D**) Immunohistochemical staining displays co-localization of platelets (CD41-positive stained red area) and infiltrated inflammatory cells (dark stained nuclei). (**E**) Representative left ventricular sections showing accumulation of Evans Blue in the infarct segments, and quantitative data indicate a marked elevation of Evans Blue content in the IZ. $n = 4$/group. $*P < 0.001$ compared with non-infarct zone (NIZ). (**F**) Splenectomy did not affect Evans Blue content in the infarct myocardium. $n = 7$–8/group.

monocyte activation [3–6], may form a potential therapeutic target.

It remains unclear exactly how splenic platelet release is initiated and how platelets are activated. Swirski et al. [26] provided evidence for elevated levels of circulating angiotensin II in mediating splenic release of monocytes following AMI. In patients or mice with AMI, circulating levels of catecholamines increased by 2–3-fold. This finding may provide clues for mechanisms 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 [28–31,44,47]. Thus, elevated levels of angiotensin II and catecholamines may jointly promote splenic platelet release. Furthermore, dynamic change of platelet size and PMC differ between infarcted human patients and mice, suggesting that these parameters are influenced by routine medications. In keeping with a previous report [48], 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$_{12}$ receptors. We previously observed in infarcted mice that blockade of P2Y$_{12}$ by clopidogrel and prasugrel inhibited platelet activation measured by P-selectin expression [5,9]. Recent studies have shown that myocardial necrosis post-MI evokes release of a variety of molecules termed damage-associated molecular patterns (DAMPs) [49]. Through binding to pattern recognition receptors such as Toll-like receptors (TLR), DAMPs activate innate immunity as well as platelets [49,50]. Indeed, Semeraro et al. [50] identified extracellular histones as the potent activator of platelets through TLRs. 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.

Our individual drug testing revealed diverse influences by perindopril, atenolol and clopidogrel on platelet parameters. Increment in platelet size was blunted by perindopril only, whereas
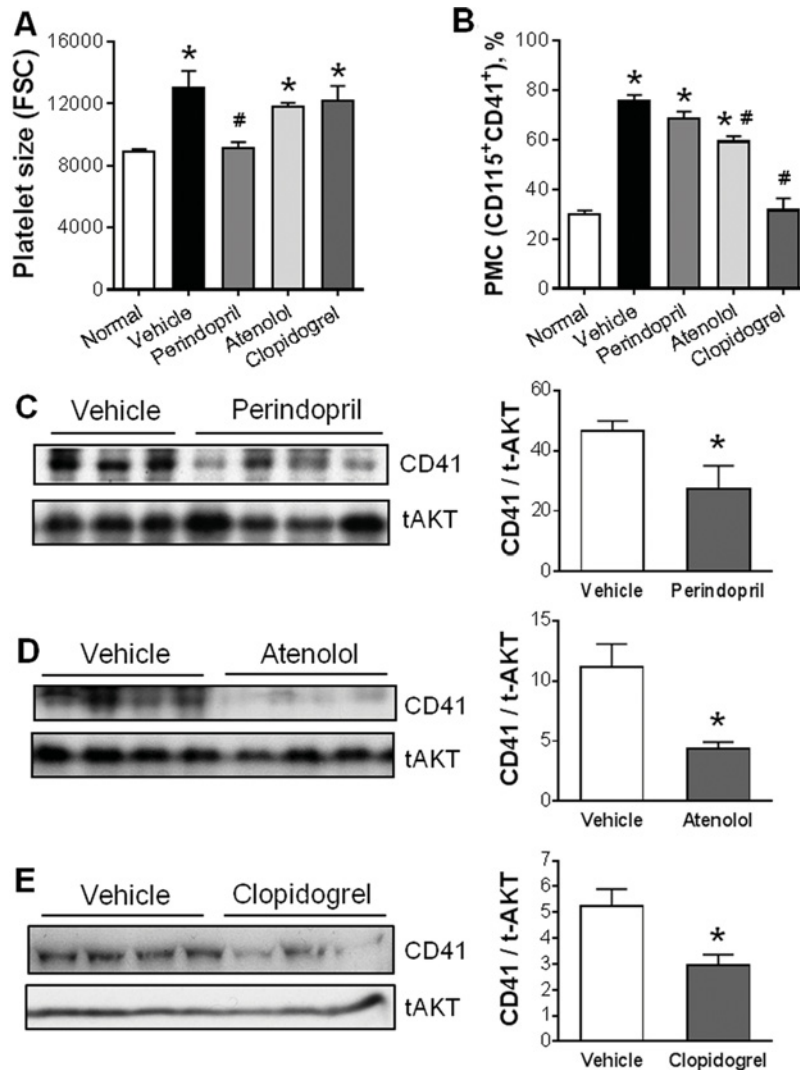
Figure 7 Effects of pharmacological inhibition of ACE, β_1-adrenergic receptor or platelet P2Y$_{12}$ receptor on platelet parameters in mice at 24 h after MI

Treatment with perindopril, but not atenolol or clopidogrel, blunted the increase in circulating platelet size (**A**). Treatment with atenolol and clopidogrel partially or largely inhibited PMC in circulating blood (**B**). All drugs tested attenuated CD41 abundance (platelet accumulation) in the infarct myocardium (**C–E**). $n = 5$–8/group. *$P<0.05$ compared with normal (A, B) or vehicle (C–E) group, #$P<0.05$ compared with vehicle group (A, B).

PMC was inhibited by both atenolol and clopidogrel. Interestingly, all three drugs were effective in inhibiting platelet accumulation in the infarct myocardium. These findings imply that current medications may inhibit at different stages of the cascade from splenic release to myocardial accumulation of monocytes/platelets (Figure 8). We compared MPV of our patients with or without pre-use of β-blocker, ACEI/ARB, aspirin or statins (Table 1) and found a lack of significant influence by drug pre-use (results not shown). Our clinical data, however, is limited by insufficient sample size. Further prospective study with adequate sample size is required to address these questions from clinical aspects.

Our previous [5] and the current study have provided histological evidence for co-existence of platelets and leucocytes within the infarct zone (IZ). Two possibilities may explain the extra-vascular platelet accumulation in the infarct region: (1) a 'piggy back' mechanism by which leucocytes conjugated with platelets migrate into the infarct region, and (2) microvascular leakiness allowing platelets to enter into myocardial interstitium. The latter is supported by our findings of leaky microvasculatures and presence of intra-myocardial haemorrhage in the infarct myocardium. Although having no effect on permeability of microvessels of infarcted tissues, splenectomy-reduced regional accumulation of inflammatory cells and platelets apparently due to reduced number and activity of spleen-derived monocytes and platelets.

In mice with MI, we observed beneficial effects by treatment with the drugs or splenectomy measured as platelet parameters and cardiac inflammation and injury. These treatments similarly suppressed platelet size, PMC or cardiac platelet content, implying at least in part platelet-dependent mechanism(s).
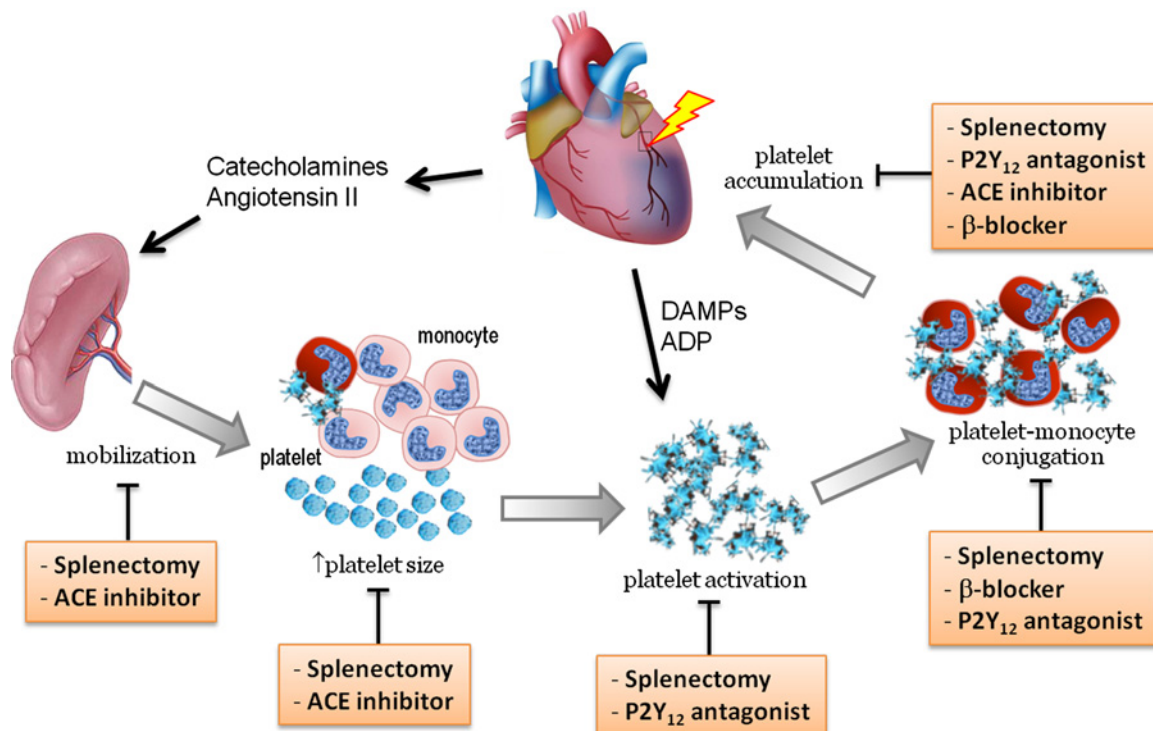
Figure 8   Scheme depicting the potential role of splenic platelets and monocytes in inflammatory response after MI
AMI rapidly induces neurohormonal activation measured by increased levels of catecholamines and angiotensin II [58], and also release of DAMPs and ADP from ischaemic tissues [49,50]. These factors evoke splenic release of platelets and monocytes [26,27], platelet activation and PMC occurring within the spleen and in the circulation. Activated circulating inflammatory cells and platelets then infiltrate into the jeopardized myocardium promoting regional inflammation with subsequent exacerbation of myocardial injury. In the present study, the events of this cascade are inhibited by the interventions tested, particularly by splenectomy. Notably, splenectomy also limited infarct size and cardiac dilatation at the acute phase. All test drugs reduced platelet accumulation in the infarct myocardium.

Numerous studies have demonstrated beneficial effects of treatment with ACEI, β-blocker, P2Y antagonists in the setting of AMI, including reduced extent of infarct size, dysfunction, arrhythmias or mortality [51–56]. In the current study, our findings of platelet-related parameters that reflect the pro-inflammatory action provide an additional mechanism for the efficacy of these drugs. Large platelets with increased activities, including PMC, are associated with adverse outcomes post-MI likely through increased risk of microthrombi or microvascular damage (e.g. no-reflow) [12,14,16–18] and enhanced regional inflammatory responses. An escalated inflammation following MI is known to damage various components of the myocardium, not only cardiomyocytes, but also ECM and microvessels, with resultant worsening of the ultimate myocardial injury and cardiac remodelling. In this context, we previously showed in the mouse MI model that treatments targeting inflammatory molecules (MMP, cytokines) or platelets ameliorated the extent of LV remodelling albeit infarct size was unchanged [5,34,57].

In conclusion, we documented that mobilization of splenic platelets is responsible for a rapid increase in circulating MPV following AMI. We have delineated a chain of events involving splenic platelet/monocyte release, increase in size and activity of circulating platelets, increase in PMC in both peripheral blood and the spleen, and myocardial accumulation of platelets and leucocytes (Figure 8). Thus, the spleen acts as a key organ in the systemic inflammatory response after AMI. The benefits achieved by splenectomy or use of pharmacological inhibitors imply that the 'cardiosplenic axis' forms a therapeutic target.

CLINICAL PERSPECTIVES

- Acute MI is characterized by early increase in circulating platelet size that is associated with adverse cardiovascular events. The inflammatory property of platelets, largely through interaction with leucocytes, has been well characterized. The reason for large platelets in circulation and its relationship with cardiac inflammation post-MI remains unclear. Using the mouse model of MI, we demonstrated release of splenic platelets that is related to increased platelet-monocyte conjugation in the circulation and inflammatory infiltration into the myocardium.

- Importantly, splenectomy abolished the increase in circulating platelet size and attenuated the extent of cardiac inflammation, effects accompanied by limitation of both infarct size and cardiac remodeling at the acute phase. Pharmacological inhibition of ACE, β-AR or platelet P2Y$_{12}$ receptor had diverse effects on increment of circulating platelet size or PMC, but

consistently reduced the abundance of platelets in the infarct myocardium.

- Our study provides a mechanism explaining the acute increase in circulating platelet size in patients at the time of hospital admission by demonstrating mobilization of the splenic platelet store. The splenic release of platelets and monocytes upon AMI contributes significantly to the systemic and cardiac inflammation and forms a therapeutic target.

AUTHOR CONTRIBUTION

Xiao-Ming Gao, Xiao-Lei Moore, Yang Liu contributed to concept and study design, data generation, analysis and interpretation of data, and critical writing or revising the intellectual content. Li-Ping Han, Yidan Su, Alan Tsai, Qi Xu, Ming Zhang, Helen Kiriazis, Xin-Yu Wang, Gavin Lambert contributed to data generation. Wei Gao, Anthony Dart, Xiao-Jun Du contributed to concept, study design and research fund, data generation, analysis and interpretation of data, critical writing the intellectual content.

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