Elevated platelet–leukocyte complexes are associated with, but dispensable for myocardial ischemia–reperfusion injury

Christopher Starz · Carmen Härdtner · Maximilian Mauler · Bianca Dufner · Natalie Hoppe · Katja Krebs · Carolin Anna Ehler · Julian Merz · Timo Heidt · Peter Stachon · Dennis Wolf · Christoph Bode · Constantin von zur Muehlen · Wolfgang Rottbauer · Meinrad Gawaz · Daniel Duerschmied · Florian Leuschner · Oliver Borst · Dirk Westermann · Ingo Hilgendorf

Received: 23 August 2022 / Revised: 18 October 2022 / Accepted: 8 November 2022 / Published online: 16 November 2022

© The Author(s) 2022

Abstract

Aims P-selectin is an activatable adhesion molecule on platelets promoting platelet aggregation, and platelet–leukocyte complex (PLC) formation. Increased numbers of PLC are circulating in the blood of patients shortly after acute myocardial infarction and predict adverse outcomes. These correlations led to speculations about whether PLC may represent novel therapeutic targets. We therefore set out to elucidate the pathomechanistic relevance of PLC in myocardial ischemia and reperfusion injury.

Methods and results By generating P-selectin deficient bone marrow chimeric mice, the post-myocardial infarction surge in PLC numbers in blood was prevented. Yet, intravital microscopy, flow cytometry and immunohistochemical staining, echocardiography, and gene expression profiling showed unequivocally that leukocyte adhesion to the vessel wall, leukocyte infiltration, and myocardial damage post-infarction were not altered in response to the lack in PLC.

Conclusion We conclude that myocardial infarction associated sterile inflammation triggers PLC formation, reminiscent of conserved immunothrombotic responses, but without PLC influencing myocardial ischemia and reperfusion injury in return. Our experimental data do not support a therapeutic concept of selectively targeting PLC formation in myocardial infarction.

Keywords Platelet–leukocyte complexes · Myocardial infarction · Ischemia/reperfusion injury · Inflammation · P-selectin

Abbreviations

AAR Area at risk
ADP Adenosindiphosphate
AMI Acute myocardial infarction
ANOVA One-way analysis of variance
AP Angina pectoris
ATP Adenosintriphosphate

Bl6J Short for C57Bl/Bl6J
CT Cycle treshold
CCL C-C motif ligand
CXCL C-X-C motif ligand
FACS Fluorescent activated cell sorter
FS Fractional shortening
GP Glycoprotein
hsTnT High-sensitive Troponin T
i.p. Intraperitoneal

Christopher Starz and Carmen Härdtner have contributed equally to this work.

Ingo Hilgendorf
ingo.hilgendorf@uniklinik-freiburg.de

1 Department of Cardiology and Angiology, University Heart Center Freiburg-Bad Krozingen, Faculty of Medicine, University of Freiburg, Freiburg, Germany
2 Department of Internal Medicine II, University of Ulm, Ulm, Germany
3 Department of Cardiology and Cardiovascular Medicine, University Hospital Tuebingen, Tuebingen, Germany
4 Department of Cardiology and Angiology, Faculty of Medicine, University of Heidelberg, Mannheim, Germany
5 Department of Medicine III, Faculty of Medicine, University of Heidelberg, Heidelberg, Germany
6 InflaMyoCard Research Consortium, The Baden-Wuerttemberg Ministry of Science, Research and Arts, Stuttgart, Germany
7 Institute for Experimental Cardiovascular Medicine, Faculty of Medicine, University Heart Center Freiburg-Bad Krozingen, University of Freiburg, Freiburg, Germany
### Basic Research in Cardiology (2022) 117:61

| i.v. | Intravenous |
| I/R | Ischemia and reperfusion |
| IRS | Ischemia/reperfusion injury |
| IVM | Intravital microscopy |
| KO | Knock out |
| KOWT | Knock out in wild-type chimera |
| LAD | Left anterior descending |
| LVEDD | Left-ventricular end-diastolic diameter |
| LVEF | Left-ventricular ejection fraction |
| LVESD | Left ventricular end-systolic diameter |
| MFI | Mean fluorescence intensity |
| MIP | Macrophage inflammatory protein |
| Mono | Monocyte |
| MΦ | Macrophage |
| neutro | Neutrophile |
| NSTEMI | Non-ST-elevating myocardial infarction |
| PBS | Phosphate buffered saline |
| PF4 | Platelet factor 4 |
| PLC | Platelet leukocyte complexes |
| PLT | Platelet/thrombocyte |
| PMC | Platelet monocyte complexes |
| PNC | Platelet neutrophile complexes |
| PRP | Platelet-rich plasma |
| Rantes | Regulated and Normal T-cell Expressed and Secreted (= CCL5) |
| RBC | Red blood cell |
| ROS | Reactive oxygen species |
| STEMI | ST-elevating myocardial infarction |
| TIMI | Thrombolysis in Myocardial Infarction |
| TNFα | Tumor necrosis factor alpha |
| TTC | 2,3,5-Triphenyltetrazoliumchloride |
| WBC | White blood cell |
| WT | Wild type |
| WTKO | Wild type in knockout chimera |
| WTWT | Wild type in wild-type chimera |

### Introduction

Improving outcomes and preserving cardiac tissue in patients with acute myocardial infarction (AMI) represents one of the most challenging unmet medical needs in cardiovascular care to date.

The gold standard in treating AMI is the timely restoration of myocardial blood flow to minimize ischemic injury, but it comes at a price: Reperfusion injury. Myocardial ischemia and reperfusion (I/R) injury manifests itself in reperfusion arrhythmia, myocardial stunning, microvascular obstructions, and cardiomyocyte cell death, responsible for up to 50% of the final infarct size [59]. Unfortunately, most therapeutic interventions directed against myocardial I/R injury have failed to show benefits in clinical trials to date [9, 46]. A rare exception may be P-selectin antagonism in patients undergoing percutaneous coronary intervention for non-ST-segment elevation MI, which blunted post-procedural troponin and creatine kinase-myocardial band rises in blood as surrogates of reduced myocardial damage [53], but clinical endpoints have not been tested in a large follow-up trial.

The formation of platelet–leukocyte complexes (PLC) largely depends on the binding of platelet-derived P-selectin to leukocyte-derived P-selectin glycoprotein ligand 1 (PSGL-1) [14, 27, 42, 46]. In platelets, P-selectin is stored intracellularly in alpha granules and is transferred to the cell surface upon platelet activation [39]. Increased frequencies of circulating PLC, platelet–monocyte (PMC) and platelet–neutrophil complexes (PNC) in particular, have been recorded in patients with AMI or unstable angina [18, 38, 43]. Elevated numbers of circulating PMC represent early markers of AMI [38]. Their frequencies correlate with troponin levels [60] and predict future cardiovascular events within 2 years following AMI [61]. Experimental studies, ex vivo, suggest that platelet binding to monocytes and neutrophils can stimulate proinflammatory responses [11, 14] and facilitate microvascular obstructions in vivo [22, 24, 40]. Taken together, PLC are postulated to contribute directly to myocardial I/R injury, and to represent a therapeutic target [25, 32]. In this study, we challenge the hypothesis of a direct causal involvement of PLC in myocardial I/R injury in vivo.

### Methods

#### Animals

Male C57BL/6J (WT) and P-selectin-deficient B6;129S7-Selptm1Bay/J (KO) mice were provided by the Jackson Laboratories (Maine, USA). Eight-week-old mice from both genotypes were lethally irradiated and reconstituted with $10^7$ WT or KO bone marrow cells, respectively. Hereby, we created three different types of bone marrow chimeras: WT in WT (WTWT), KO in WT (KOWT), and WT in KO (WTKO). Euthanasia was performed by CO$_2$ inhalation. All animal procedures were approved by the Animal Care Committee of the University Freiburg and regional councils.

#### Blood collection and cell counts

Murine blood was obtained by facial vein puncture and collected in 10 µL Enoxaparin per 100 µL blood. Larger volumes were gained after euthanasia with CO$_2$ and cardiac cannulation. Leukocytes were counted manually, using a Neubauer counting chamber and diluting with red blood cell lysis buffer 1:20 (Thermo Fisher Scientific, Waltham, MA, USA). Standardized blood counts for leukocytes, platelets,
and erythrocytes were performed by the University Central Laboratory.

**Plasma**

For plasma generation, blood was centrifuged in two steps at 1500×g at room temperature for 5 min, collecting the supernatant. Plasma was immediately shock frozen in liquid nitrogen and stored at −20 °C until further processing.

**Platelet-rich plasma (PRP)**

Murine blood was diluted 1:1 with Tyrode's buffer and 0.3% enoxaparin, centrifuged, and the supernatant transferred the supernatant (PRP) to a new reaction tube. PRP was always kept at 37 °C. For measuring surface marker expression, PRP was stimulated with 10 µL 200 µM adenosine diphosphate (ADP) (Merck KGaA, Darmstadt, Germany) for 30 min and analyzed by flow cytometry.

**Flow cytometry**

Heparinized blood was diluted 1:6 with PBS. Diluted blood was used for antibody staining. After 15 min of incubation at 37 °C, blood was lysed and fixed with BD® Lysis/Fix™ (BD Bioscience, Becton Dickinson, Heidelberg, Germany).

Heart tissues were enzymatically digested [collagenases I and IX, hyaluronidase as well as DNase I (Sigma-Aldrich, St. Louis, Missouri, USA)] and heart cell suspensions are processed through a 40 µm strainer using PBS/0.5% BSA/1% FCS. Cells were stained with specific antibodies. For endothelial cell isolation, we minced aortas and digested them with collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase I (Sigma-Aldrich, St. Louis, Missouri, USA). Antibodies used are listed in Supplemental Table 1. For ROS detection, blood cells were incubated with 2′,7′-dichlorofluorescin diacetate (DCFDA, Merck KGaA, Darmstadt, Germany) prior to antibody staining.

Flow cytometry analysis was performed with a BD FACS Canto II (BD Bioscience, San Diego, CA, USA).

**Imaging flow cytometry**

Imaging flow cytometry analysis of murine blood samples was performed at the Image Stream Core Facility (Tübingen, Germany) with an Imagestream® X Mark II Imaging Flow Cytometer (Merck KGaA, Darmstadt, Germany). In this approach, fluorescent antibody surface staining is combined with intracellular phalloidin staining of actin following cell permeabilization with 0.1% Triton X (both obtained from Merck KGaA, Darmstadt, Germany).

Antibodies used are listed in the supplemental methods (Supplemental Table 1).

**Histology**

The infarcted areas of isolated hearts were embedded in TissueTek® O.C.T.™ (OCT) (Sakura Fintek, Tokyo, Japan), frozen at −20 °C and cut into 4 µm sections. Neutrophils were identified by anti-Ly6G rat anti-mouse antibody binding (BD, New Jersey, USA). For overall tissue, morphology hematoxylin and eosin staining was performed.

**High-sensitive troponin T (hsTnT) levels**

Plasma was diluted 1:15 with PBS, and hsTnT levels were determined by automated electrochemiluminescence analysis according to the detailed protocol from Andrassy et al. [1].

**Myocardial ischemia–reperfusion (I/R) injury**

In anesthetized mice, the left anterior descending (LAD) coronary vessel was obstructed for 35 min and then reperfused again. Sham operated mice underwent only thoracotomy followed by 35 min of anesthesia [buprenorphine (0.1 mg/kg) and isoflurane inhalation]. 24 h after I/R, mice were euthanized, hearts were flushed with isotonic saline and excised for further processing. Postoperative analgesia was performed with buprenorphine (0.1 mg/kg) subcutaneously every 6–8 h.

**Echocardiography**

Transthoracic echocardiography was performed on isoflurane anesthetized mice with a Vevo 3100 ultrasound device with an MX550D linear probe (Visualsonics, Toronto, Canada). Left-ventricular ejection fraction was measured using the Simpson method.

**Peritonitis model**

For sterile peritonitis, mice were injected with 2 mL 4% thioglycollate intraperitoneally (i.p.) (Merck KGaA, Darmstadt, Germany). After 4 h, mice were euthanized, and peritoneal lavage was performed by injecting and aspirating 5 mL PBS. Cells were washed, counted (Neubauer counting chamber), and stained for flow cytometry analysis.
**A**

Blood leukocytes

- **SSC-A**
  - CD11b
  - PNC

- **Ly6G**
  - neutro

- **Ly6C**
  - PNC

- **CD41**
  - mono

- **CD115**

**B**

- **PNC**
  - % neutrophils
  - 0, 6, 24, 72 hours

- **PMC**
  - % monocytes
  - 0, 6, 24, 72 hours

**C**

- **brightfield**
  - PNC
  - 7 μm

- **CD41**
  - Ly6G
  - merged
  - F-actin

- **brightfield**
  - PMC
  - 7 μm

- **CD41**
  - CD115
  - merged
  - F-actin

**D**

- **PNC**
  - r = 0.569
  - p = 0.0267
  - % neutrophiles
  - troponin T [ng/mL]

- **PMC**
  - r = 0.822
  - P = 0.000517
  - % monocytes
  - troponin T [ng/mL]

Ischemia

- sham
- 35 min
- 45 min
- permanent
**Intravital microscopy**

Mice were stimulated by injecting 10 g/kg tumor necrosis factor alpha (TNFα) i.p. 4 h prior to intravital microscopy. Following ketamine (100 mg/kg) and xylazine (100 mg/kg) anesthesia and rhodamine injection (i.p.), we exposed the mesenteric venules and recorded up to 12 30-s clips per mouse.

Rhodamine intravital microscopy was performed with an Axiotech vario 100 HD microscope, a AxioCam Mrm digital camera, and Colibri 2 light source (all Zeiss, Oberkochen, Germany).

For two-color intravital microscopy, mice were injected intravenously with X488/anti-GPIbβ (emfret Analytics, Eibelstadt, Germany) to label platelets and with anti-Gr1-PE (Bio Legend) to label neutrophils and classical monocytes 15 min prior to image recording. Analysis was performed with a Nikon® FN-S2N fluorescence microscope, Hamamatsu Orca-05G digital camera and Nikon Intenslight C-HGFIE.

**TTC**

After excision of the heart and proximal aorta, the LAD was occluded again at the site of the suture. The hearts were perfused with Monolight blue 5% followed by further rinsing with 0.9% NaCl and rapid freezing. Frozen hearts were sliced and incubated for 5 min at 37 °C in 2,3,5-triphenyl-tetrazolium chloride (TTC). After 24 h at 4 °C, heart slices were photo-documented and analyzed for area at risk (AAR) and infarct area.

**qRT-PCR**

Frozen infarcted heart tissue was defrosted, homogenized, and processed according to the instructions of the Qiagen RNeasy® Mini Kit (Qiagen, Düsseldorf, Germany). Hereby generated RNA was further transcribed into cDNA using the High-Capacity cDNA Reverse Transcription cDNA Kit (Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer’s instructions. cDNA transcription was performed with a Taq-Man probes and a CFX96 Touch Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Taq-Man probes used are listed in supplemental Table 2. Target gene expression levels were calculated using the 2^−ΔΔCT method with β-actin as the housekeeping gene. Results per mouse and target gene were interpreted and visualized relative to mean target gene expression levels in WTWT chimeras.

**Statistical analysis**

Results are shown as Mean ± standard error of the mean (SEM). Results are declared as significant with a p value ≤ 0.05. The choice of statistical calculation method was based on criteria such as normal distribution and the number of groups to be compared. When comparing two normally distributed groups with mutually independent measurements, the unpaired t test (also Student’s t test) was applied. Multiple t test was used to compare the PCR data. Due to the multiple testing, a Bonferroni correction was applied. If there were more than two groups to be compared with measurements that were independent of each other, statistical significance was tested with the one-way analysis of variance (ANOVA) with Holm–Sidak posthoc testing. The Kruskal–Wallis test was used as a non-parametric test with Dunns post hoc testing. Statistical analysis was performed using Excel 2010 (Microsoft, Redmond, Washington, USA) and Prism 6 (GraphPad Software, San Diego, California, USA). When considering more than one influencing variable, statistical significance was tested using the two-way analysis of variance (ANOVA), assuming a normal distribution. Sidak’s post hoc test was also applied. Bivariate correlations of PMC or PNC levels with plasma troponin levels were calculated using GraphPad Software 2021 (San Diego, CA, USA).

**Results**

**PMC and PNC surge in blood following myocardial ischemia and reperfusion in mice**

We identified and quantified complexes of platelets with monocytes and neutrophils in blood following fluorescent antibody staining and flow cytometry in mice (Fig. 1A). PMC and PNC formation was visualized and confirmed by imaging flow cytometry (Fig. 1C). Frequencies of PMC and PNC circulating in blood following 35 min of myocardial...
A
platelets (ADP)
Endothelial cells (TNFα)
DWT
DWT
DWT

B
dead
blood
live

C
intravital
rolling

D
blood

WTWT
KOWT
WTKO

WTWT
KOWT
WTKO

WTWT
KOWT
WTKO

WTWT
KOWT
WTKO
ischemia with reperfusion for 6, 24, and 72 h were compared to those quantified in healthy and sham operated mice (Fig. 1B). PMC and PNC formation peaked and more than tripled after 24 h of reperfusion and returned to pre-procedural levels at the 72 h reperfusion time point (Fig. 1B). PNC and PMC fractions correlated positively with troponin T levels measured 24 h post 35 min or 45 min of myocardial ischemia with reperfusion, or post-permanent LAD ligation, respectively (Fig. 1D). These data are in line with reports in patients with AMI, in which PLC frequencies increase transiently after coronary revascularization and in which troponin T levels correlate with PMC frequencies [3, 38]. Since 35 min of myocardial I/R injury induced reproducible and sufficient troponin elevations with significant increases in circulating PLC numbers, we used this model to explore the functional role of PLC in myocardial I/R injury.

Platelets and leukocytes within PLC are relatively activated compared to freely circulating cells

First, we set out to quantify relative cell surface protein expression levels of leukocyte and platelet activation markers in platelet-bound monocytes and neutrophils compared to unbound blood cells in C57Bl6 mice (Suppl. Fig. 1A). Direct comparison was complicated by the fact that CD41 expression levels on platelets within PLC appeared on average lower than levels detected in non-leukocyte-bound platelets. Since CD41 is constitutively expressed on platelets, as confirmed by ex vivo ADP stimulation of whole blood (Suppl. Fig. 1B) and in line with image flow cytometry data (Fig. 1C), we concluded that only one or few platelets bind per leukocyte, while higher numbers of non-leukocyte-bound platelets can form aggregates among each other cumulatively expressing higher mean levels of CD41 (Suppl. Fig. 1A, C). To control for the bias introduced by differences in platelet numbers forming platelet–leukocyte and platelet–platelet aggregates, we normalized platelet activation marker expression levels in PLC and non-leukocyte-bound platelets to the mean fluorescence intensity (MFI) levels of CD41 in the respective populations. Higher levels of P-selectin (CD62P), glycoproteins (GP) Ib, IIb/IIIa and VI were detected on platelets forming complexes with monocytes and neutrophils compared to those not bound to leukocytes. Reactive oxygen species were highest in leukocytes binding platelets compared to non-complexed leukocytes and platelets (Suppl. Fig. 1D). These data indicate that activated platelets exposing high levels of surface P-selectin preferentially engage in PLC formation.

Inhibiting PLC formation by deleting P-selectin on platelets

To test the functional impact of PLC in a mouse model of myocardial I/R, we had to generate a PLC-deficient model first. P-selectin (CD62P) is exclusively expressed on activated platelets and endothelial cells (EC) [7] and binds to PSGL-1 on leukocytes mediating PLC formation and endothelial rolling and tethering [16, 34]. Therefore, a global P-selectin-deficient mouse model is not suitable to test the role of PLC selectively, prompting us to explore the potential of irradiation bone marrow chimeras. We generated three types of chimeras: (I) Irradiated C57Bl6 wild-type (WT) mice reconstituted with WT bone marrow cells (WTWT group), (II) irradiated WT mice reconstituted with P-selectin deficient (KO) bone marrow cells (KOWT group), and (III) irradiated KO mice reconstituted with WT bone marrow cells (WTKO group). In the WTWT irradiation control group, P-selectin is readily detectable on ADP-stimulated platelets and TNFα-stimulated aortic endothelial cells, whereas in KOWT mice, P-selectin is lost on platelets but not endothelial cells, and vice versa in WTKO mice (Fig. 2A). PLC formation was evaluated by blood flow cytometry, and leukocyte rolling and adhesion to the vessel wall were imaged by intravital microscopy of mesenteric venules in the three groups (Fig. 2B–D). Both PNC and PMC fractions were reduced by at least 70% in KOWT mice in which P-selectin is lost selectively in platelets, but not in WTKO chimeras. In contrast, leukocyte rolling along the vessel wall was impaired in WTKO chimeras in which P-selectin is lost on endothelial cells. Intriguingly, leukocyte rolling and attachment remained unaffected in KOWT chimeras despite the lack of PLC.

Inhibiting PLC formation by deleting P-selectin on platelets
Lack of PLC does not impair leukocyte infiltration to sites of sterile inflammation

Although platelets are generally thought to facilitate leukocyte attachment and infiltration into tissues, our initial intravital microscopy experiment suggested that platelet-derived P-selectin and PLC formation do not play a relevant role in this process. To substantiate our finding, we performed two-color intravital microscopy with anti-Gr-1-PE (labeling both Ly6Chigh monocytes and neutrophils) and anti-GP1b-Alexa488 (labeling platelets) antibodies visualizing rolling PLC in vivo. Similar to the differences observed in peripheral blood, the fraction of monocytes and neutrophils rolling on or adhering to mesenteric venules which carried platelets was significantly reduced in KOWT chimeras compared to WTWT chimeras despite equal leukocyte and platelet counts (Fig. 3A, Suppl Fig. 2).

Next, we induced thioglycolate-elicited sterile peritonitis to test leukocyte infiltration in the presence or absence of PLC. 4 h post-intraperitoneal thioglycolate injection, total monocyte and neutrophil numbers were similar in blood of WTWT and KOWT chimeras, whereas PMC and PNC fractions remained significantly reduced in KOWT chimeras (Fig. 3B). Yet, the numbers of neutrophils and macrophages retrieved from the peritoneal lavage did not differ between the groups (Fig. 3C). Taken together, our experiments indicate that P-selectin-mediated leukocyte binding and PLC formation do not support inflammation-driven leukocyte extravasation.

Elevated PLC are associated with, but dispensable for myocardial I/R injury

As shown in Fig. 1B, experimental myocardial I/R injury leads to a transient increase in circulating PLC numbers peaking after 24 h of reperfusion. In KOWT chimeras, however, the surge in PMC and PNC numbers observed in WTWT chimeras post-infarction does not occur (Fig. 4A) despite comparable monocyte and neutrophil numbers (Fig. 4B). In accord with our previous observations in the intravital microscopy and peritonitis models, monocyte, neutrophil, and macrophage numbers in myocardial infarct tissues, as determined by enzymatic tissue digestion and flow cytometric analysis, were similar in KOWT and WTWT chimeras, although PMC and PNC fractions were relatively reduced both in blood and myocardial tissue cell suspensions of KOWT chimeras (Fig. 4C). Immunohistologic staining for Ly6G+ neutrophils confirmed this finding.

Troponin T levels in plasma of KOWT and WTWT chimeras collected 24 h post-myocardial infarction showed no
difference between the groups (Fig. 4D). In line, both the area at risk (AAR) and the infarct size determined by TTC staining were indistinguishable in both groups (Fig. 4E). Echocardiography revealed comparable declines in cardiac function at 21 days post-myocardial infarction in both groups (Fig. 4G). Expression levels of proinflammatory and preresolving genes in infarct tissues after 24 h of reperfusion documented only marginal differences between WTWT and KOWT chimeras (Fig. 4G). Taken together, although PLC increase as a result of myocardial I/R injury, their loss, reciprocally, does not impede myocardial injury in the short and long term.

**Discussion**

Associations of elevated circulating PLCs with myocardial infarct size and adverse long-term outcomes in patients have led to the hypothesis that these two phenomena are causally related in the sense that PLC aggravate cardiac injury [47, 60, 61]. Experimental studies in the past have lent support to this hypothesis.

For instance, simultaneous infusion of neutrophils and platelets during reperfusion of ischemic isolated guinea pig and rat hearts impaired cardiac function [20, 29, 49]. In line, blocking PNC-formation attenuated these adverse cardiac effects [21]. Specifically, blocking P-selectin–P-selectin glycoprotein ligand 1 (PSGL 1) interaction reduced PLC formation, neutrophil infiltration and experimental myocardial ischemia reperfusion injury [5, 30, 41]. However, in contrast to our bone marrow chimera model, these approaches did not selectively target platelet-derived P-selectin, and thus affected endothelial P-selectin-mediated leukocyte rolling, directly.

At the site of experimental vascular injury, platelets cover the area of endothelial denudation, and platelet-derived P-selectin can imitate endothelial-derived P-selectin’s role to capture PSGL-1 expressing leukocytes and facilitate monocyte/macrophage accumulation in the neointima [33]. Similar processes may be at play during atherogenesis, when P-selectin deficiency in platelets partially protects from atherosclerotic plaque development in ApoE-deficient mice, whereas endothelial P-selectin deficiency has a more profound atheroprotective effect [8]. In myocardial infarction, however, myocardial injury develops in a large ischemic area distal to the site of an occluding coronary artery plaque, and leukocyte infiltration into the infarct tissue occurs via post-capillary venules distinct from arterial recruitment in atherosclerosis [15, 35]. Hence, mesenteric venule intravital microscopy and sterile peritonitis are valid complementary models for gaining insight into inflammatory cell recruitment into infarct tissue. In our work, intravital microscopy, peritoneal lavage analysis, and flow cytometric or immunohistochemical staining of myocardial infarct tissue showed unanimously that loss of platelet P-selectin neither slowed cell recruitment nor protected from myocardial ischemia and reperfusion injury despite a significant reduction in circulating, rolling, and tissue infiltrating PLC. In line, Rosenkranz and colleagues, using P-selectin-deficient bone marrow chimeras, reported similar kidney infiltrating leukocyte counts in a murine model of glomerulonephritis [45].

Our findings suggest a reverse causation between PLC and myocardial ischemia and reperfusion injury. Larger infarcts, as indicated by the surrogate parameter cardiac troponin, lead to the release of tissue injury related danger signals that activate platelets. Upon activation, platelets expose P-selectin forming complexes with neutrophils and monocytes. To this effect, PLC represents a biomarker of cardiac injury rather than a culprit.

Observational studies documented that elevated PLC correlate with inadequate reperfusion post-STEMI-percutaneous coronary intervention due to microvascular obstructions as assessed by reduced Thrombolysis in Myocardial Infarction (TIMI) flow and myocardial blush grade [4, 44]. A causal relationship, however, has not been established experimentally. While our study clearly indicates that PLC do not contribute to myocardial ischemia and reperfusion injury, the murine transient LAD ligation model may not fully recapitulate all pathophysiological aspects of interventional revascularization in STEMI patients, including those that lead to microvascular obstructions. Moreover, our study does not dispute a critical role of platelets in fostering leukocyte activation and extravasation by other means than P-selectin-dependent PLC formation, or in different disease contexts [48].

The biological role of PLC formation upon danger signal recognition may be immunologic. Platelets can scavenge, immobilize, and aggregate bacteria, and thus facilitate their clearance and destruction through phagocytes and neutrophil extracellular trap formation [50].

Platelets appear to circulate in the bloodstream as “surveillance drones” to be the first cellular actors on site in the context of vascular injury or tissue inflammation [31]. Healthy endothelium prevents platelet activation and aggregation, in part via nitric oxide and prostacyclin secretion. In the context of endothelial injury and activation, these inhibitory effects are compromised and platelets bind to subendothelial extracellular matrix via direct platelet GP VI–collagen interaction or von-Willebrand factor (vWF)-mediated binding to platelet GP Ib/IX/V surface complex [26, 28]. In addition, multiple soluble damage-associated molecular patterns (DAMPs) similar to pathogen-associated molecular patterns (PAMPs) activate platelets via pattern recognition receptors (Toll-like receptors, NOD-like receptors, and receptor for advanced glycation end products) [2,}

© Springer
13, 17, 51, 54]. Stimulated platelets activate surface integrins and release chemokines and serotonin from granules [54]. Platelet serotonin, in turn, stimulates neutrophil rolling and adhesion to the endothelium and neutrophil activation, including increased PNC formation [12, 36]. Platelet-secreted chemokines include CXCL1, platelet factor 4 (PF4/
Platelet–leukocyte complexes form in the setting of inflammation, including sterile inflammation in the context of myocardial infarction. Preventing P-selectin-dependent complex formation, however, does not improve myocardial salvage or mitigate inflammation and leukocyte transmigration. Although PLC levels may serve as a biomarker of the magnitude of myocardial ischemia and reperfusion injury, our study suggests that targeting PLC formation, specifically, does not hold a therapeutic promise in the context of myocardial infarction.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00395-022-00970-3.

Acknowledgements We thank Prof. Schenke-Layland from the University of Tuebingen for providing access to Image Stream microscopy.

Author contributions CS, CH, and IH conceived the study, interpreted results, and wrote the manuscript. CS, CH, MM, BD, NH, KK, CAE, and JM performed animal experiments and statistical analysis. IH, TH, PS, DW CB, CM, and DW helped to interpret the results and advised regarding further investigations, IH, WR, MG, DD, PL, and OB provided resources, helped to perform experiments, and interpreted the results. All authors refined the manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. This work was supported by the Baden-Wuerttemberg Ministry of Science, InflaMyoCard research consortium to C.V.M., W.R., M.G., and M. DEAL. This work was supported by the Baden-Wuerttemberg Ministry of Science, InflaMyoCard research consortium to C.V.M., W.R., M.G., F.L. O.B., I.H.; the German Research Foundation (HI1573/2, SFB1425 Grant 422691945) to I.H.; the MOTIVATE doctoral program, Faculty of Medicine, University of Freiburg to C.S.

Data availability Data are available from the corresponding author upon reasonable request.

Declarations Conflict of interest The authors declared that they have no conflict of interest.

Ethical approval All animal procedures were approved by the Animal Care Committee of the University Freiburg and regional councils in accordance with the directive 2010/63/EU of the European Parliament.
Consent to participate Not applicable.

Consent for publication Not applicable.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Andrassy M, Volz HC, Maack B, Schuessler A, Gitsioudis G, Hofmann N, Loachachewin D, Wienbrandt AR, Kaya Z, Bierhaus A, Giannitsis E, Katus HA, Korosoglou G (2012) HMGB1 is associated with atherosclerotic plaque composition and burden in patients with stable coronary artery disease. PLoS One 7(12):e52081. https://doi.org/10.1371/journal.pone.0052081

2. Andrews RK, Berradt MC (2004) Platelet physiology and thrombosis. Thromb Res 114(5–6):447–453. https://doi.org/10.1016/j.thromres.2004.07.020

3. Aparicio-Vergara M, Shiri-Sverdlov R, de Haan G, Hofker MH (2010) Bone marrow transplantation in mice as a tool for studying the role of hematopoietic cells in metabolic and cardiovascular diseases. Atherosclerosis 213(2):335–344. https://doi.org/10.1016/j.atherosclerosis.2010.05.030

4. Aurigemma G, Scalone G, Tomai F, Altamura L, de Persio G, Hofmann N, Loachachewin D, Wienbrandt AR, Kaya Z, Bierhaus A, Giannitsis E, Katus HA, Korosoglou G (2012) HMGB1 is associated with atherosclerotic plaque composition and burden in patients with stable coronary artery disease. PLoS One 7(12):e52081. https://doi.org/10.1371/journal.pone.0052081

5. Andrews RK, Berradt MC (2004) Platelet physiology and thrombosis. Thromb Res 114(5–6):447–453. https://doi.org/10.1016/j.thromres.2004.07.020

6. Aparicio-Vergara M, Shiri-Sverdlov R, de Haan G, Hofker MH (2010) Bone marrow transplantation in mice as a tool for studying the role of hematopoietic cells in metabolic and cardiovascular diseases. Atherosclerosis 213(2):335–344. https://doi.org/10.1016/j.atherosclerosis.2010.05.030

7. Blann AD, Nadar SK, Lip GYH (2003) The adhesion molecule P-selectin and cardiovascular disease. Eur Heart J 24(24):2166–2179. https://doi.org/10.1093/eurheartj.24.24.2166

8. Burger PC, Wagner DD (2003) Platelet P-selectin facilitates atherosclerotic lesion development. Blood 101(7):2661–2666. https://doi.org/10.1182/blood-2002-07-2209

9. D’Ascenzo F, Moretti C, Omedè P, Cerrato E, Cavallero E, Er F, Presutti DG, Colombo F, Crimi G, Conrotto F, Dinicolantonio JJ, Chen S, Prasad A, Biondi Zoccai G, Gaia F (2014) Cardiac remote ischemic preconditioning reduces perioperative myocardial infarction for patients undergoing percutaneous coronary interventions: a meta-analysis of randomised clinical trials. EuroIntervention 9(12):1463–1471. https://doi.org/10.4244/EIJV9I12A244

10. Deppermann C, Kubes P (2018) Start a fire, kill the bug: the role of platelets in inflammation and infection. Innate Immun 24(6):335–348. https://doi.org/10.1177/1753425918789255

11. Dib PRB, Quirino-Teixeira AC, Merij LB, Pinheiro MBM, Rozini SV, Andrade KB, Hotz ED (2020) Innate immune receptors in platelets and platelet-leukocyte interactions. J Leukoc Biol 108(4):1157–1182. https://doi.org/10.1002/jlb.4MR0620-701R

12. Duerschmdied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, Cifuni SM, Mauler M, Cicko S, Bader M, Idzko M, Bode C, Wagner DD (2013) Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. Blood 121(6):1008–1015. https://doi.org/10.1182/blood-2012-06-437392

13. Estevez B, Du X (2017) New concepts and mechanisms of platelet activation signaling. Physiology (Bethesda) 32(2):162–177. https://doi.org/10.1152/physiol.00020.2016

14. Finsterbusch M, Schrottmaier WC, Kral-Pointer JB, Szalma M, Assinger A (2018) Measuring and interpreting platelet-leukocyte aggregates. Platelets 29(7):677–685. https://doi.org/10.1080/09537104.2018.1430358

15. Frangogiannis N (2002) The inflammatory response in myocardial infarction. Cardiovasc Res 53(1):31–47. https://doi.org/10.1016/S0008-6363(01)00434-5

16. Frenette PS, Johnson RC, Hynes RO, Wagner DD (1995) Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci USA 92(16):7450–7454. https://doi.org/10.1073/pnas.92.16.7450

17. Furie B, Furie BC (2005) Thrombus formation in vivo. J Clin Invest 115(12):3355–3362. https://doi.org/10.1172/JCI26987

18. Furman MI, Barnard NR, Krueger LA, Fox ML, Shilale EA, Lessard DM, Marchese P, Frelinger AL, Goldberg AJ, Michelbacher DE (2010) Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. J Am Coll Cardiol 38(4):1002–1006. https://doi.org/10.1016/j.jacc.2009.10.018

19. Gleissner CA, von Hundelshausen P, Ley K (2008) Platelet chemokines in vascular disease. Arterioscler Thromb Vasc Biol 28(11):2019–2027. https://doi.org/10.1161/ATVBAHA.108.169417

20. Götz AK, Zahler S, Stumpf P, Welsch U, Becker BF (2005) Intracoronary formation and retention of micro aggregates of leukocytes and platelets contribute to postischememic myocardial dysfunction. Basic Res Cardiol 100(5):413–421. https://doi.org/10.1007/s00392-005-0540-9

21. Habazettl H, Hauusch K, Kupatt C (2000) Effects of endothelium/leukocytes/platelet interaction on myocardial ischemia–reperfusion injury. Z Kardiol 89(Suppl 9):IX92–IX95. https://doi.org/10.1007/s00392-000-7003-8

22. Heusch G (2019) Coronary microvascular obstruction: the new frontier in cardioprotection. Basic Res Cardiol 114(6):45. https://doi.org/10.1007/s00395-019-0756-8

23. Hidari KI, Weyrich AS, Zimmerman GA, McEver RP (1997) Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils. J Biol Chem 272(45):28750–28756. https://doi.org/10.1074/jbc.272.45.28750

24. Kleinbongard P, Heusch G (2022) A fresh look at coronary microembolization. Nat Rev Cardiol 19(4):265–280. https://doi.org/10.1038/s41569-021-00632-2

25. Köhler D, Straub A, Weissmüller T, Faigle M, Bender S, Lehmann R, Wendel H-P, Kurz J, Walter U, Zacharowski K, Rosenberger P (2011) Phosphorylation of vasodilator-stimulated phosphoprotein prevents platelet-neutrophil complex formation and dampens myocardial ischemia-reperfusion injury. Circulation 123(22):2579–2590. https://doi.org/10.1161/CIRCULATIONAHA.110.014555
26. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL (1996) Platelets and shear stress. Blood 88(5):1525–1541
27. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B (1989) PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell 59(2):305–312. https://doi.org/10.1016/0092-8674(89)90292-4
28. Leebeeck FWG, Eikenboom JCI (2016) Von Willebrand’s disease. N Engl J Med 375(21):2067–2080. https://doi.org/10.1056/NEJMra16101561
29. Lefer DJ, Flynn DM, Buda AJ (1996) Effects of a monoclonal antibody directed against P-selectin after myocardial ischemia and reperfusion. Am J Physiol 270(1 Pt 2):H88–H98. https://doi.org/10.1152/ajpheart.1996.270.1.H88
30. Lefer AM, Campbell B, Scalia R, Lefer DJ (1998) Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion: role of selectins. Circulation 98(13):1322–1328. https://doi.org/10.1161/01.CIR.98.13.1322
31. Li JL, Zarbock A, Hidalgo A (2017) Platelets as autonomous responders: implications for hemostasis and immune surveillance. J Exp Med 214(8):2193–2204. https://doi.org/10.1084/jem.201708789
32. Linden MD, Jackson DE (2010) Platelets: pleiotropic roles in atherosclerosis and arteriosclerosis. Int J Biochem Cell Biol 42(11):1762–1766. https://doi.org/10.1016/j.biocel.2010.07.012
33. Manka D, Forlow SB, Sanders JM, Hurwitz D, Bennett DK, Green SA, Ley K, Sarembock IJ (2004) Critical role of platelet P-selectin in the response to arterial injury in apolipoprotein-E-deficient mice. Arterioscler Thromb Vasc Biol 24(6):1124–1129. https://doi.org/10.1161/01.ATV.00000127619.04687.4f
34. Massberg S, Enders G, Leiderer R, Eisenmenger S, Vestweber D, Krombach F, Messmer K (1998) Platelet-endothelial cell interactions during ischemia/reperfusion: the role of P-selectin. Blood 92(2):507–515
35. Mauersberger C, Hintzolder J, Schunkert H, Kessler T, Sager HB (2021) Where the action is—leukocyte recruitment in athero-sclerosis. Front Cardiovasc Med 8:103394. https://doi.org/10.3389/fcvm.2021.813984
36. Mauler M, Herr N, Schoenichen C, Witsch A, Marchini T, Härdtner C, Koentges C, Kienle K, Ollivier V, Schell M, Dorner L, Wippel C, Stalhmann D, Normann C, Bugger H, Walther P, Wolf D, Ahrens I, Lämmermann T, Ho-Tin-Noé B, Ley K, Bode C, Hilgendorf I, Duerschmied D (2019) Platelet serotonin aggravates myocardial ischemia/reperfusion injury via neutrophil degranulation. Circulation 139(7):918–931. https://doi.org/10.1161/CIRCULATIONAHA.118.039342
37. McEvoy RP (2015) Selectins: Initiators of leucocyte adhesion and signalling at the vascular wall. Cardiovasc Res 107(3):331–339. https://doi.org/10.1093/cvr/crv154
38. Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI (2001) Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. Circulation 104(13):1533–1537. https://doi.org/10.1161/01.hc3801.095588
39. Morrow DA (ed) (2006) Cardiovascular biomarkers: pathophysiology and disease management. Humana Press, Totowa (Contemporary Cardiology)
40. Niccoli G, Montone RA, Ihanez B, Thiele H, Crea F, Heusch G, Bulluck H, Hausenloy DJ, Berry C, Stiermaier T, Camici PG, Eitel I (2019) Optimized treatment of ST-elevation myocardial infarction. Circ Res. https://doi.org/10.1161/CIRCRESAHA.119.315344
41. Oostingh GJ, Pozgajova M, Ludwig RJ, Krahn T, Boehncke W-H, Nieswandt B, Schön MP (2007) Diminished thrombus formation and allleviation of myocardial infarction and reperfusion injury through antibody- or small-molecule-mediated inhibition of selectin-dependent platelet functions. Haematologica 92(4):502–512. https://doi.org/10.3324/haematol.10741
42. Ostrovsky L, King AJ, Bond S, Mitchell D, Lorant DE, Zimmerman GA, Larsen R, Niu XF, Kubes P (1998) A juxtaglomerular mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. Blood 91(8):3028–3036
43. Ott I, Neumann FJ, Gawaz M, Schmitt M, Schömig A (1996) Increased neutrophil-platelet adhesion in patients with unstable angina. Circulation 94(6):1239–1246. https://doi.org/10.1161/01.cir.94.6.1239
44. Ren F, Mu N, Zhang X, Tan J, Li L, Zhang C, Dong M (2016) Increased platelet–leukocyte aggregates are associated with myocardial no-reflow in patients with ST elevation myocardial infarction. Am J Med Sci 352(3):261–266. https://doi.org/10.1016/j.amjms.2016.05.034
45. Rosenkranz AR, Mendrick DL, Cotran RS, Mayadas TN (1999) P-selectin deficiency exacerbates experimental glomerulonephritis: a protective role for endothelial P-selectin in inflammation. J Clin Invest 103(5):649–659. https://doi.org/10.1172/JCI15183
46. Sarma J, Laan CA, Alam S, Jha A, Fox KAA, Dransfield I (2002) Increased platelet binding to circulating monocytes in acute coronary syndromes. Circulation 105(18):2166–2171. https://doi.org/10.1161/01.CIR.0000015700.27754.6f
47. Schanje N, Bode C, Duerschmied D (2019) Platelet contributions to myocardial ischemia/reperfusion injury. Front Immunol 10. https://doi.org/10.3389/fimmu.2019.01260
48. Schrottmaier WC, Mussbacher M, Salzmann M, Assinger A (2020) Platelet–leukocyte interplay during vascular disease. Atherosclerosis 307:109–120. https://doi.org/10.1016/j.atherosclerosis.2020.04.018
49. Seligmann C, Leitsch T, Kusus M, Bock A, Schimmer M, Simsek Y, Daniel WG (2003) PMN/platelets coinfused in guinea pig hearts exposed to low-flow ischemia have no additive cardiopressive effect. J Vasc Res 40(6):501–508. https://doi.org/10.1159/000074890
50. Stark K, Massberg S (2021) Interplay between inflammation and thrombosis in cardiovascular pathology. Nat Rev Cardiol 18(9):666–682. https://doi.org/10.1038/s41569-021-00552-1
51. Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S (2000) The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br J Haematol 110(4):925–934. https://doi.org/10.1046/j.1365-2141.2000.02208.x
52. Sukirman L (2021) Soluble P-selectin and correlation with Prothrombin Fragment 1 + 2 in myeloid malignancies in Cipto Mangunkusumo general hospital. Thromb J 19(1):51. https://doi.org/10.1186/s12959-021-00307-5
53. Tardif J-C, Tanguay J-F, Wright SR, Duchatelle V, Petroni T, Grégoire JC, Ibrahim R, Heinenon TM, Robb S, Bertrand OF, Cournoyer D, Johnson D, Mann J, Guertin M-C, L’Allier PL (2013) Effects of the P-selectin antagonist inclacumab on myocardial infarction, reperfusion, secretion and procoagulant activity. Br J Haematol 160(4):925–934. https://doi.org/10.1111/bjh.13492
54. Thomas MR, Storey RF (2015) The role of platelets in inflammation. Thromb Haemost 114(3):449–458. https://doi.org/10.1160/TH14-12-1067
55. Wang Y, Gao H, Shi C, Erhardt PW, Pavlovsky A, Soloviev DA, Bledzka K, Ustinov V, Zhu L, Qin J, Munday AD, Lopez J, Plow E, Simon DI (2017) Leukocyte integrin Mac-1 regulates thrombosis via interaction with platelet GPIIbα. Nat Commun 8:15559. https://doi.org/10.1038/ncomms15559
56. Weyrich AS, Elstad MR, McEver RP, McIntyre TM, Moore KL, Morrissey JH, Prescott SM, Zimmerman GA (1996) Activated platelets signal chemokine synthesis by human monocytes. J Clin Invest 97(6):1525–1534. https://doi.org/10.1172/JCI118575

57. Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA (1995) Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-xB translocation. J Clin Invest 95(5):2297–2303. https://doi.org/10.1172/JCI117921

58. Woollard KJ, Kling D, Kulkarni S, Dart AM, Jackson S, Chindusting J (2006) Raised plasma soluble P-selectin in peripheral arterial occlusive disease enhances leukocyte adhesion. Circ Res 98(1):149–156. https://doi.org/10.1161/01.RES.0000199295.14073.69

59. Yellon DM, Hausenloy DJ (2007) Myocardial reperfusion injury. N Engl J Med 357(11):1121–1135. https://doi.org/10.1056/NEJMra071667

60. Zhang S-Z, Jin Y-P, Qin G-M, Wang J-H (2007) Association of platelet–monocyte aggregates with platelet activation, systemic inflammation, and myocardial injury in patients with non-st elevation acute coronary syndromes. Clin Cardiol 30(1):26–31. https://doi.org/10.1002/clc.2

61. Zhou X, Liu X-L, Ji W-J, Liu J-X, Guo Z-Z, Ren D, Ma Y-Q, Zeng S, Xu Z-W, Li H-X, Wang PP, Zhang Z, Li Y-M, Benefield BC, Zawada AM, Thorp EB, Lee DC, Heine GH (2016) The kinetics of circulating monocyte subsets and monocyte-platelet aggregates in the acute phase of ST-elevation myocardial infarction: associations with 2-year cardiovascular events. Medicine (Baltimore) 95(18):e3466. https://doi.org/10.1097/MD.0000000000003466