Inhibition of Platelet GPVI Protects Against Myocardial Ischemia–Reperfusion Injury

Christina Pachel,* Denise Mathes,* Anahi-Paula Arias-Loza, Wolfram Heitzmann, Peter Nordbeck, Carsten Deppermann, Viola Lorenz, Ulrich Hofmann, Bernhard Nieswandt, Stefan Frantz

Objective—The objective of this study was to investigate the effects of platelet inhibition on myocardial ischemia–reperfusion (IR) injury.

Approach and Results—Timely restoration of coronary blood flow after myocardial infarction is indispensable but leads to additional damage to the heart (myocardial IR injury). Microvascular dysfunction contributes to myocardial IR injury. We hypothesized that platelet activation during IR determines microvascular perfusion and thereby the infarct size in the reperfused myocardium. The 3 phases of thrombus formation were analyzed by targeting individual key platelet-surface molecules with monoclonal antibody derivatives: (1) adhesion (anti-glycoprotein [GP]-Ib), (2) activation (anti-GPVI), and (3) aggregation (anti-GPIIbIIIa) in a murine in vivo model of left coronary artery ligation (30 minutes of ischemia followed by 24 hours of reperfusion). Infarct sizes were determined by Evans Blue/2,3,5-triphenyltetrazolium chloride staining, infiltrating neutrophils by immunohistology. Anti-GPVI treatment significantly reduced infarct size versus control, whereas anti-GPIib or anti-GPIIbIIIa antibody fragments showed no significant differences. Mechanistically, anti-GPVI antibody–mediated reduction of infarct size was not because of impaired Ca2+ signaling or platelet degranulation because aggregation by an anti-GPIb or anti-GPIIbIIIa antibody significantly reduces infarct size. The reduction of the infarct size is primarily based on an improved microperfusion after anti-GPVI antibody treatment. (Arterioscler Thromb Vasc Biol. 2016;36:629-635. DOI: 10.1161/ATVBAHA.115.305873.)

Key Words: GPVI collagen receptor

Antisite of vascular injury, the subendothelial extracellular matrix is exposed to the blood and triggers sudden platelet activation and the formation of a fibrin containing thrombus. This process is essential to prevent excessive posttraumatic blood loss, but if it occurs inappropriately, for example, at sites of atherosclerotic plaque rupture, it can lead to vessel occlusion and ischemic infarction. In the clinical context of myocardial infarction, timely reopening of an occluded coronary artery by pharmacological thrombolysis or coronary intervention constitutes an effective therapeutic mean to limit cardiac damage. However, restoration of blood flow causes further cardiac damage, the so-called reperfusion injury (RI). The mechanisms of myocardial RI are not fully understood; thus, it is possible that thrombus formation by platelets in reperfused microvessels could contribute to this process. Furthermore, thrombus fragmentation by thrombolysis or coronary balloon angioplasty might add to the occlusion of microvessels by distal embolization. Additionally, platelets release factors that potentially increase RI without mechanical obstruction of microvessels. Activated...
platelets secrete granule contents, including chemokines (eg, RANTES [regulated on activation, normal T cell expressed and secreted], platelet factor 4), cytokine-like factors (eg, interleukin-1β, CD40-ligand, β-thromboglobulin), and coagulation factors (eg, Factor V, Factor XI, PAI-1 [plasminogen activator inhibitor-1], plasminogen), which activate the endothelium and enhance leukocyte adhesion, extravasation, and the formation of interstitial edema. Activated platelets also bind to leukocytes in the blood and interact with endothelial cells by direct cell–cell contacts.1,2

Basically, thrombus formation can be divided into 3 distinct phases:1 (1) adhesion, (2) activation, and (3) aggregation of platelets with the platelet membrane receptors glycoprotein (GP)Ib-V-IX, GPVI, and GPIbIIIa being involved in all these steps: platelets can adhere to hypoxic endothelial cells by binding of their GP Ib receptor to von Willebrand factor (vWF) on the endothelial surface. Locally exposed subendothelial matrix proteins, such as collagens, facilitate the attachment of platelets by the GP VI receptor. Subsequent platelet activation involves an increase in cytosolic Ca2+ concentration, which is mediated by the stromal interaction molecule 1 (STIM1), a key regulatory protein of the stromal interaction molecule 1 (STIM1), and a calcium release–activated calcium channel protein 1 (Orai-1) as principal Ca2+ entry channel.4 These processes lead to the activation of platelet GPIbIIIa, the release of granules, and finally platelet aggregation.

Therapeutics that interfere with platelet aggregation, like acetylsalicylic acid, ADP (adenosine-di-phosphate) receptor antagonists (clopidogrel), antagonists of the major platelet integrin (IIb)3 (GPIIbIIIa), and phosphodiesterase inhibitors,7 are well established in the clinical setting of acute coronary syndrome, limiting thrombus formation. Although being effective, they are all linked to an increased risk of life-threatening bleeding events. A better understanding of the mechanisms leading to platelet activation in the context of myocardial RI might help to identify new therapeutic targets to limit platelet-induced RI without further increasing the risk for clinically relevant bleeding. In the present study, we hypothesized that platelet activation contributes to RI, and we aimed to identify targets that reduce ischemia–reperfusion (IR) injury with minimal or comparable risk of bleeding to the current therapeutic standards.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
GPVI-Dependent Myocardial Reperfusion Injury Involves Neither SOCE Nor Platelet Degranulation

Next, we aimed at identifying the mechanism for infarct size reduction by anti-GPVI treatment. The activation of GPVI on platelets leads to a massive increase in cytosolic Ca\(^{2+}\) concentrations via SOCE, a process that essentially depends on the endoplasmic reticulum Ca\(^{2+}\) sensor molecule STIM1 and the Ca\(^{2+}\) channel protein Orai1. However, in Stim1\(^{−−}\) or Orai1\(^{−−}\), bone marrow chimeric mice INF/AAR was not altered (wild-type versus Stim1\(^{−−}\) versus Orai1\(^{−−}\): INF/AAR: 48.4±6.4\% versus 53.1±14.7\% versus 67.5±11.5\%; P=n.s.; [n=5]; Figure 3B). Downstream of GPVI activation, platelet α-granules and dense granules degranulate. Neither Nbeal2\(^{−−}\) mice, lacking α-granules, nor Unc13d\(^{−−}\) mice, displaying abolished platelet-dense granule secretion and reduced α-granule release, were protected from IR injury (wild-type versus Nbeal2\(^{−−}\) versus Unc13d\(^{−−}\): INF/AAR: 35.2±5.5\% versus 39.5±5.1\% versus 46.7±7.9\%; P=n.s.; [n=6]; Figure 3C). In conclusion, GPVI-dependent promotion of IR injury does not require SOCE or the release of α- and dense granule content.

Enhanced Microperfusion in Anti-GPVI-Treated Mice

Reduced microvascular obstruction might explain the protective effects of anti-GPVI treatment. To test for this, we measured microperfusion after 30 minutes of ischemia and 24 hours of reperfusion using a fluorescent microsphere protocol in mice treated with PBS (control), anti-GPVI antibody (platelet inhibition, reduced infarct size), and anti-GP Ib (platelet inhibition, no reduced infarct size; Figure III in the online-only Data Supplement). To measure the total perfusion, blue fluorescent microspheres were injected. To assess the area at risk, red fluorescent microspheres were injected after the reocclusion of the coronary artery. The area at risk (no red microspheres) was similar in all groups. However, perfusion of the reperfused ischemic area (blue microspheres) was significantly increased in animals treated with anti-GPVI (PBS versus anti-GPVI versus anti-GP Ib: 0.74±0.10 versus 1.02±0.10 versus 0.90±0.08; [n=5]; Figure 4). Thus, despite similar platelet inhibiting capacity, only anti-GPVI, but not anti-GP Ib, treatment increased the perfusion of the previously ischemic area after reperfusion.

Platelet Inhibition Induces Myocardial Hemorrhage

A severe side effect of platelet inhibition could be intramyocardial hemorrhage after myocardial infarction, which might potentially abolish protective antithrombotic effects. Because intramyocardial hemorrhage is difficult to quantify by immunohistology, we visualized hemorrhage by magnetic resonance imaging in a subset of mice (Figure IV in the online-only Data Supplement). To measure the total perfusion, blue fluorescent microspheres were injected. To assess the area at risk, red fluorescent microspheres were injected after the reocclusion of the coronary artery. The area at risk (no red microspheres) was similar in all groups. However, perfusion of the reperfused ischemic area (blue microspheres) was significantly increased in animals treated with anti-GPVI (PBS versus anti-GPVI versus anti-GP Ib: 0.74±0.10 versus 1.02±0.10 versus 0.90±0.08; [n=5]; Figure 4). Thus, despite similar platelet inhibiting capacity, only anti-GPVI, but not anti-GP Ib, treatment increased the perfusion of the previously ischemic area after reperfusion.
Anti-GPVI and Anti-GPIb Antibody–Treated Mice Show Decreased Numbers of Leukocytes in the Reperfused Myocardium

Multiple interaction mechanisms between platelets and leukocytes lead to recruitment and activation of inflammatory cells to sites of platelet activation. We analyzed leukocytes in reperfused myocardium by immunohistology and found the numbers of neutrophils in the myocardium to be reduced both in anti-GPVI- and anti-GPIb–treated mice (Figure 5).

As an additional approach, we performed flow cytometric analysis of intramyocardial leukocytes. Accordingly, we found a reduced amount of CD45+ leukocytes infiltrating the ischemia-reperfused zone of anti-GPVI- and anti-GPIb–treated mice (PBS versus anti-GPVI versus anti-GPIb: 3.9±1.7% versus 1.3±0.6% versus 0.9±0.2%; [n=26]; Figure 6A), but no differences in the frequencies of infiltrating CD3+ T cells (Figure 6B) and their CD4+ (Figure 6C) and CD8+ subsets (Figure 6D). Taken together, anti-GPVI and anti-GPIb were both able to reduce neutrophil infiltration after IR.

Discussion

In the present study, we investigated the hypothesis that pathological platelet activation contributes to myocardial RI. To test this hypothesis, we studied the 3 main phases of platelet-dependent thrombus formation individually: (1) initial adhesion, (2) activation, and (3) firm adhesion/aggregation.

The initial capture of flowing platelets was inhibited with an anti-GP Ib antibody that interrupted the interaction of GPIb-V-IX with vWF immobilized on exposed collagens. Neither infarct sizes nor microperfusion were affected by this approach. Platelet aggregation, spreading on the extracellular matrix of the injured vessel wall, and thrombus formation are mediated by the binding of integrin αIIbβ3 (GPIIIbIIa) to its ligands fibrinogen, fibronectin, and vWF. This process was inhibited using an anti-GPIIbIIIa antibody. This approach did not significantly alter infarct size.

After the initial capturing of flow engaging platelets, the collagen receptor GPVI can bind to its ligand and initiate cellular activation. We studied the role of GPVI in myocardial RI by using an anti-GPVI antibody that induces irreversible downregulation of the GPVI receptor on the surface of circulating platelets. This is different from a previous approach targeting GPVI-mediated platelet activation in myocardial RI: Schönberger et al administered the dimeric soluble fusion protein GPVI-Fc Revacept, consisting of the extracellular part of the human platelet collagen receptor GPVI and the Fc domain of human IgG1. Mechanistically, this recombinant GPVI-Fc binds to activated endothelium mainly via vitronectin and inhibits platelet/endothelial interaction, whereas the pretreatment with the anti–mouse GPVI antibody JAQ1 used in the present study leads to irreversible depletion of GPVI on circulating platelets, resulting in abolished responses of the cells to collagen. Administration of Revacept reduced infarct size, preserved cardiac function, and attenuated platelet-mediated inflammatory responses after transient myocardial ischemia. Reduction of infarct size after the administration of GPVI-Fc was associated with reduced GPVI-induced platelet degranulation and release of proinflammatory cytokines. Revacept has already been tested in a first-in-human study (phase I) and appeared to be safe and well-tolerated with a clear dose-dependent pharmacokinetic profile.

In our present study, we studied a new approach to target GPVI in myocardial IR, using the anti-GPVI antibody JAQ1. Here, GPVI is irreversibly downregulated on circulating platelets by antibody-induced ectodomain shedding. Using this anti-GPVI antibody, we found significantly reduced INF/AAR and improved myocardial microperfusion after RI, providing a promising alternative to Revacept. Both approaches...
underline the therapeutic relevance of the GPVI pathway for myocardial IR injury.

How Does Anti-GPVI Treatment Confer Protection?
The immune system has long been recognized as an important mediator of cardiac IR. Neutrophils infiltrate the myocardium within minutes after ischemia, and they mediate ischemic inflammation and aggravate infarct size. Siminiak et al described a cross talk between neutrophils and platelets that can result in capillary plugging in the coronary microcirculation and thereby contribute to the no reflow phenomenon. Further support for a link between platelets and neutrophils in cardioprotection comes from a study by Takaya et al, examining Fc receptor-γ chain–deficient mice. The Fc receptor-γ chain is noncovalently associated with GPVI in the platelet membrane and is essential for the expression of and signal transduction by this receptor. Takaya et al showed that Fc receptor-γ chain–deficient mice were protected from myocardial IR injury. Myeloperoxidase activity, an indicator for neutrophil infiltration, and the adhesion of platelets with neutrophils were reduced in Fc receptor-γ chain–deficient mice, whereas CD11b expression on neutrophils was not altered.

After GPIb- and GPVI inhibition, we found reduced numbers of neutrophils in the infarct zone. Reduced neutrophil numbers correlated with smaller infarct sizes for the anti-GPVI-treated but not for anti-GPIb-treated mice. Therefore, the postulated cross talk between neutrophils and platelets does not fully explain the protective effect of anti-GPVI antibody treatment.

Importantly, we observed significantly improved microperfusion in mice treated with anti-GPVI—but not in mice treated with anti-GPIb antibody—in the ischemic area after reperfusion. An explanation of improved microperfusion after GPVI inhibition might be given by a recent publication of Mammadova-Bach et al. Here, GPVI was identified as a platelet receptor for polymerized fibrin, which can amplify thrombin generation and recruit circulating platelets to clots. This publication highlights the central function of GPVI not only in the phase of platelet activation, but also in the phase of thrombus growth and the stabilization of thrombi. Therefore, the function of GPVI as a receptor for polymerized fibrin might explain the significantly improved microperfusion in anti-GPVI-treated mice in comparison to anti-GPIb treatment, which only affects the initial capture of flowing platelets. A significant limitation of the anti-GPVI treatment was an increased prevalence of myocardial hemorrhage, which might be responsible for the observation that the infarct sparing effect did not lead to an immediately improved ejection fraction compared with the control treatment group.
Finally, the cross talk between blood platelets and immune cells can also be influenced by microparticles. These are membrane-coated vesicles, which can be released by many cell types during activation and apoptosis. Platelet-derived microparticles were identified as a contributing factor to rheumatoid arthritis, where their generation crucially depends on GPVI. This is in accordance with a previous study showing GPVI activation to increase the formation of platelet-derived microparticles in whole blood samples. To date, little is known about the role of microparticles in whole blood samples. It seems plausible that the GPVI inhibition might affect platelet-derived microparticle formation in an IR setting, but this link was not assessed in our current study.18

To further decipher the mechanism of GPVI-mediated reduction of infarct size, series of platelet-specific knockout mice (Stim1−/−, Orai1−/−, Nbeal2−/−, Unc13d−/−) were used. In platelets, moderate and transient elevation in cytosolic calcium [Ca2+]i levels mediates shape change, integrin (Ibβ3) activation, thromboxane formation, and secretion of granule contents, whereas high and sustained intracellular calcium [Ca2+]i rises are required for the procoagulant response. Here we hypothesized that GPVI-dependent promotion of RI might be related to those components. However, our experiments clearly showed that STIM1/Orai1-dependent SOCE in platelets is not required for this pathological process.

The content of platelet-dense granules (δ-granules) and α-granules promote platelet aggregation, thrombus formation, and stability (by means of calcium, serotonin, ADP, and ATP release; δ-granules) or mediate adhesiveness and inflammation (by mediators, such as vWF, fibrinogen, P-selectin, interleukin-1β, interleukin-8, α-granules). Activation of GPVI leads to the degradation of platelet α- and dense granules. Nbeal2−/− mice lack α-granules in megakaryocytes and platelets. Mice carrying an inactivating point mutation in the Unc13d gene (Unc13d−/− mice), leading to the absence of Munc13-4 in platelets, display abolished platelet-dense granule secretion and reduced α-granule release. To date, little is known about the granules’ contribution to thromboinflammatory processes like myocardial RI. Here we found no significant alterations in infarct sizes in either Nbeal2−/− or Unc13d−/− mice, arguing against a significant role of degranulation in the pathophysiology of myocardial IR injury.

What Explains the Different Results of Myocardial Infarction and Stroke?

The role of platelets in ischemic stroke has been extensively studied. GPIb was identified as an attractive target for the clinical development of an anti-thromboinflammatory drug, significantly reducing cerebral infarcts. Treating mice with anti-GPIbα Fab 1 hour before and 1 hour after middle cerebral artery occlusion clearly reduced ischemic lesions compared with controls. Interaction of the GPIb-V-IX complex with vWF is transient and reduces the velocity of the cells, thereby enabling interactions between the low-affinity platelet collagen receptor, GPVI, and collagen. In acute experimental stroke, depletion of GPVI diminished the infarct volume significantly, but to a lesser extent than GPIb, whereas in our model of the ischemic and reperfused heart, solely depletion of GPVI clearly reduced INF/AAR. Although in a small subset of IR mice, intramyocardial hemorrhage occurred in anti-GPVI- and anti-GPIb-treated mice, in a model of acute experimental stroke, mice receiving anti-GPIbα Fab or anti-GPVI mAbs did not show hemorrhagic transformation by serial magnetic resonance imaging studies. Blockade of the final common pathway of platelet aggregation with anti-GPIbH1a F(ab)2 fragments dose-dependently increased the incidence of intracerebral hemorrhage after transient middle cerebral artery occlusion. This led to an improved outcome after stroke in anti-GPVI-treated animals.

Our differing findings in the myocardium might also be explained by differences in intensity of shear stress. Under conditions of low shear, such as those found in larger arteries and veins, platelet adhesion to the vessel wall mainly involves binding to fibrillar collagens, fibronectin, and laminin. Although the initial contact of the GPIb-V-IX complex with vWF is dispensable for platelet adhesion under conditions of high shear flow, GPVI initiates platelet aggregation at both high and low shear stress. Therefore, the assumed higher shear stress in the brain than in the heart might be responsible for different ways of platelet activation.

Conclusions
IR injury can be reduced by inhibiting platelet activation via GPVI. This effect is not primarily because of an anti-thrombotic effect but rather indicates that platelets impair microvascular reperfusion. Still, more experiments on the preservation of cardiac function by the anti-GPVI antibody and hemorrhage are needed. Nevertheless, our data provide an indication that anti-GPVI treatment may be a valuable approach to reduce ischemic myocardial injury as an adjunct to standard antiplatelet therapy.

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Disclosures
None.

References
beyond a better reperfusion that is reached already by platelet-inhibiting strategies currently in clinical practice. The clinical use of the platelet activation–inhibiting anti-glycoprotein-VI antibody together with reperfusion might limit myocardial injury.

References


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Inhibition of platelet glycoprotein VI (GPVI) protects against myocardial ischemia-reperfusion injury

Christina Pachel, PhD, Denise Mathes, PhD, Anahi-Paula Arias-Loza, PhD, Wolfram Heitzmann, MD, Peter Nordbeck, MD, Carsten Deppermann, PhD, Viola Lorenz, PhD, Ulrich Hofmann, MD, Bernhard Nieswandt, PhD and Stefan Frantz, MD

¶ These authors contributed equally.

Materials and Methods

Materials

Antibodies

Monoclonal antibodies were generated and modified as described previously. An overview of the antibodies and the therapeutical application scheme is given in Table 1. In all experiments using antibody treatment, an individual control group with phosphate buffered saline (PBS) treatment was studied in parallel.

<table>
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<th>dose per mouse (25-28g)</th>
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<td>anti-GPVI</td>
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<td>anti-CLEC-2</td>
<td>INU1</td>
<td>200 µg</td>
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</table>

Table 1: Platelet inhibiting antibodies.

Mice

C57BL/6J mice were purchased from The Harlan Laboratories (The Netherlands). Pld1<sup>-/-</sup>, Nbeal2<sup>-/-</sup>, Unc13d<sup>-/-</sup>, and Stim1<sup>-/-</sup> and Orai1<sup>-/-</sup> bone marrow chimeras were generated as previously described. Mice were operated 6 weeks after irradiation/ bone marrow transplantation. All recipient animals received acidified water containing 2 g/L neomycin sulfate for 2 weeks. Using C57BL/6J mice, 50 µg anti-GP Ibα F(ab) (clone p0p/B) and 100 µg anti-GP IbIIa F(ab)2 (clone JON/A) were injected retro-orbitally (r.o.) at reperfusion while 100 µg anti-GPVI (clone JAQ1) and 200 µg anti-CLEC-2 (clone INU1) were injected before ischemia-reperfusion intraperitoneally (i.p.). The anti-CLEC2 and anti-GPVI treatments were performed on day 6 before ischemia-reperfusion to allow for a normalization of the platelet count after the treatment as these antibodies lead to a drop in platelet count. Anti-CLEC2 induces a transient thrombocytopenia in the treated mice. The platelet count drops by more than 85% on day 1 and returns to normal values after 3-4 days. Anti-GPVI leads to thrombocytopenia accompanied by a down-regulation of the GPVI receptor on circulating platelets. The platelet count returns to normal values within 48 hours whereas the GPVI-deficiency lasts for 14 days.

Methods

Animals and Surgery

8-12 week old C57BL/6J or mutant mice underwent left coronary artery ligation for 30 min (ischemia), followed by 24h of reperfusion, as described elsewhere. Anesthesia was...
performed with 1.5% isoflurane. Buprenorphine was given standardized for pre-emptive analgesia. As control, sham operation was performed in the same manner, but the ligature was not closed. All animal studies were approved by the district government of Lower Franconia (“Bezirksregierung Unterfranken”).

**Measurement of infarct size per area at risk**

Infarct size per area at risk was determined by Evans Blue/ TTC staining according to the detailed protocol previously described. Briefly after 30 minutes of ischemia and 24 hours of reperfusion, the animals were intubated, the suture was reoccluded, and 5% Evans blue (Sigma) was injected in the right ventricle followed by a cardioplegic solution (10% potassium chloride). Hearts were excised, washed with NaCl, frozen for 30 minutes at -20°C in Tissue Tek (Sakura), and cut transversely into five cross-sections, including one section at the site of ligature. Sections were then incubated for 10 minutes at 37°C in 2% 2,3,5-Triphenyltetrazolium chloride (TTC, Sigma) to determine viability. After TTC staining, viable myocardium is stained red and the infarcted area appears pale. Sections were weighted and imaged with a Leica EZ4HD stereo microscope, and the area of infarction for each slice was determined by computerized planimetry (VGA software, Microsoft Excel). The size of infarction was determined by the following equations: weight of infarction = (A1×Wt1) + (A2×Wt2) + (A3×Wt3) + (A4×Wt4) + (A5 ×Wt5), where A is percent area of infarction by planimetry and Wt is the weight of each section; percentage of infarcted left ventricle = (weight of infarction/weight of LV) × 100. Area at risk (AAR) as a percentage of LV = (weight of LV – weight of LV stained blue)/ weight of LV. To account for the fact, that the infarct size varies due to vessel anatomy, performing ischemia-reperfusion, we used matched vehicle controls for all experimental series. Therefore, only one PBS-control group could be used as internal reference group for both anti-GPllb and anti-GPllbllla antibody-treated mice.

**Perfusion measurement**

Mice were anesthetized after 30 min of ischemia and 24 h of reperfusion. Total cardiac perfusion was determined by injection of 100µl 1µm Blue fluorescent 365/415 microspheres solution (Invitrogen) in 10% Bovine Albumin Serum into the beating heart. Then the suture was closed again and 50µl of a 0.2µm Red fluorescent 580/605 microspheres solution (Invitrogen) in 10% Bovine Albumin Serum was injected into the right ventricle. The area with red fluorescent microspheres is the non-ischemic area of the myocardium, confined by microthrombi. Areas without red microspheres indicate the area at risk. Blue microspheres can distribute to all intact and perfused vessels of the heart. Areas without blue microspheres determine regions of “no reflow”. The hearts were collected and embedded in Tissue Tek (Sakura). The frozen heart was cut into 30µm slices of the left ventricle. Pictures of the infarct and border zone as well as of the septum were taken with a BZ analyzer system (BIOZERO, Keyence). Green autofluorescence was used for normalization.

**Magnetic Resonance Imaging**

MRI (7 Tesla Bruker Biospec 70/ 20 scanner; Bruker Biospin, Ettlingen, Germany) was performed in vivo after 30 min of ischemia and 24 h of reperfusion as recently described. A 7 Tesla Bruker Biospec 70/ 20 scanner (Bruker Biospin, Ettlingen, Germany) was used for imaging. MRI was done under inhalation anesthesia using oxygen-enriched (1 l/ min) room air with 1.5% isoflurane (Isoflurane forene, Abbott), as recently described. After determination of the standard section planes, the imaging protocol started with an ECG-triggered multi-slice short-axis cine-FLASH (fast low-angle shot) with an in-plane image resolution of 0.117 x 0.117 and slice thickness of 1 mm, contiguously covering the whole heart. Image acquisition in cardiac T2 and T2* imaging for detection of myocardial edema and hemorrhage was performed.
in the late diastole, therefore avoiding heart motion. Measurements were done in a midventricular short-axis and 4-chamber long-axis view (field-of-view 30 x 30 mm, slice thickness 2 mm). Data post-processing was done using Matlab/IDL. Semi-quantitative evaluation of myocardial edema/hemorrhage was determined by myocardial signal intensity from the T2 and T2* maps. Ejection fraction (EF) was determined based on MRI measurements.

**Flow Cytometry**

For flow cytometry analysis of mouse hearts, previously infarcted and reperfused parts of individual hearts, which could be identified visually as appearing pale, were used. Accurate removal of blood cells was guaranteed by lysis of erythrocytes. Cardiac tissues were digested with collagenase type 2 and protease type XIV (Sigma) and minced through 40µm cell strainers (Becton Dickinson), as described elsewhere. For one staining process, about 10^6 trypan-blue negative total heart suspension cells were used. FACS buffer, in which cells were washed and antibodies were diluted, consisted of phosphate buffered saline (PBS) / 0.1% bovine serum albumin (BSA) / 0.02% sodium azide (NaN3). Unspecific binding of antibodies was avoided by blocking of Fcy II/III receptors (TruStain fcX™ (anti-mouse CD16/32) antibody; BioLegend). For extracellular staining, cell pellets were incubated with 25µl of antibody cocktails for 15 minutes (4°C). For each fluorochrome used in one panel, single stainings and unstained controls of fresh murine spleen cell suspensions were prepared for compensation. The samples were measured on a LSRII (Becton Dickinson) and analyzed by FlowJo (Tristar, Ashland OR) software.

To measure platelet count and surface expression of GPVI and CLEC-2, 50 µl of blood were collected in heparin-containing tubes and diluted 1:20 in PBS. Subsequently, platelets were stained for 15 min with saturating amounts of fluorophore-conjugated antibodies and immediately analyzed on a FACSCalibur (Becton Dickinson). To determine platelet counts of different samples the flow cytometric settings were adjusted that counting was stopped after a defined period of time (30 seconds).

Antibodies for flow cytometry are specified in Table 2.

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<td>anti-rat IgG isotype control for INU 1</td>
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<td></td>
<td>Emfret</td>
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</table>

Table 2: List of FACS antibodies.

**Immunohistology**

Formalin-fixed sections of mouse myocardium were prepared in the standard manner. Sections were consecutively labelled with rat-anti-mouse neutrophil primary antibody (clone 7/4; Linaris) and secondary biotinylated anti-rat antibody (4001; Linaris), followed by detection using a Vectastain avidin-biotin complex ABC kit, as recommended by the manufacturer (Vector Laboratories). The slides were then washed, dehydrated, and mounted for light microscopy. Images were acquired using an AxioImager.Z1 microscope (Carl Zeiss) equipped with an AxioCamMR3 camera.
Statistics
Results are presented as the mean ± standard error of mean (S.E.M.). For data collection and statistical analysis, Microsoft Excel and Graph Pad Prism software were used. The comparison between two groups was performed by two-tailed unpaired t-test and the comparison between three groups was performed by one-way ANOVA followed by post hoc Bonferroni test. For the MRI analysis, a two-tailed Fisher’s exact test (Graph Pad software) was performed. A p-value <0.05 is considered statistically significant (*). The confidence interval is 95%.
References


Supplemental Material

Inhibition of platelet glycoprotein VI (GPVI) protects against myocardial ischemia-reperfusion injury

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¶ These authors contributed equally.

Supplemental Figures

Supplemental Figure I

A
Supplemental Figure I: Control of the experimental setup - Successful depletion of GPVI from the platelet surface on day 5 after anti-GPVI antibody treatment with comparable platelet count before induction of myocardial ischemia-reperfusion.

A) On day 5 after the injection of 100µg anti-GPVI antibody in WT mice, GPVI was depleted successfully from the platelet surface versus PBS-treated control mice, measured by flow cytometry, depicted as histogram. To detect depletion of GPVI from the platelet surface in flow cytometry, a FITC-conjugated anti-rat JAQ 1 antibody (black line) versus a FITC-conjugated anti-rat IgG isotype control for JAQ 1 (grey line) was used. B) Platelet counts of resting platelets of anti-GPVI antibody treated WT mice and PBS-treated WT controls are comparable on day 5 after injection, guaranteeing comparable conditions before induction of myocardial ischemia-reperfusion (platelet count: WT: 100±14% vs. anti-GPVI: 81±18% with the mean platelet count of the WT set to 100%).
Supplemental Figure II

A

B

**Supplemental Figure II: Determination of infarct size per area at risk.**
Representative images after Evans Blue/ TTC staining of heart slices of **A)** PBS treated mice versus **B)** anti-GPVI treated mice.
Supplemental Figure III: Images of perfusion measurement with red and blue fluorescent microspheres.

**A**) Red and blue microspheres are distributed in the whole myocardium of sham operated mice. **B), C**) IR operated mice with non-ischemic area (red microspheres) and area without red microspheres, which defines the area of no perfusion (due to the performed ligation). Blue microspheres define the reperfused area of the myocardium (Figure 3B, lower arrow; Figure 3C, arrow) and the area without blue microspheres is the area of “no reflow” (Figure 3B, upper arrow).
Supplemental Figure IV: Determination of hemorrhage (Magnetic Resonance Imaging; MRI).

Representative MRI pictures of anti-GPIIbIIIa and anti-GPVI-treated mice versus sham operated mice. Hemorrhage in anti-GPIIbIIIa- and anti-GPVI-treated mice are marked by white arrows.

Supplemental Figure V: Determination of ejection fraction.
Ejection fraction (EF) was determined in anti-GPIb and anti-GPVI antibody-treated mice versus PBS control after ischemia-reperfusion and MRI, showing no statistical significant difference in EF between the groups.

Supplemental Table I

<table>
<thead>
<tr>
<th>operation</th>
<th>treatment</th>
<th>number of mice with hemorrhage per total</th>
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<tbody>
<tr>
<td>sham</td>
<td>PBS control</td>
<td>0/4 (0%)</td>
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<tr>
<td>sham</td>
<td>anti-GPIb antibody</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>sham</td>
<td>anti-GPVI antibody</td>
<td>0/2 (0%)</td>
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<tr>
<td>sham</td>
<td>anti-GPIIbIIIa antibody</td>
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</tr>
<tr>
<td>pooled sham</td>
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<tr>
<td>IR</td>
<td>PBS control</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>IR</td>
<td>anti-GPIb antibody</td>
<td>3/9 (33%)</td>
</tr>
<tr>
<td>IR</td>
<td>anti-GPVI antibody</td>
<td>2/9 (22%)</td>
</tr>
<tr>
<td>IR</td>
<td>anti-GPIIbIIIa antibody</td>
<td>1/6 (17%)</td>
</tr>
</tbody>
</table>

Supplemental Table I: MRI assessment of a subset of ischemia-reperfusion or sham operated mice.