Diminished thrombus formation and alleviation of myocardial infarction and reperfusion injury through antibody- or small-molecule-mediated inhibition of selectin-dependent platelet functions

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ABSTRACT

Background and Objectives

P-selectin has been implicated in important platelet functions. However, neither its role in thrombus formation and cardiovascular disorders nor its suitability as a therapeutic target structure is entirely clear.

Design and Methods

Platelet aggregation was assessed in complementary *in vitro* settings by measurements of static aggregation, standardized aggregometry and dynamic flow chamber assays. Degradation of aggregates was also analyzed under flow conditions using video microscopy. *In vivo*, platelet rolling in cutaneous venules was assessed by intravital microscopy in wild-type mice treated with selectin-blocking compounds as well as in P-selectin-deficient mice. $FeCl_3$-induced arterial thrombosis was studied by intravital microscopy in untreated mice or mice treated with an inhibitor of selectin functions. Finally, inhibition of selectin functions was studied in an ischemia/reperfusion injury model in rats.

Results

Antibody- or small-molecule-mediated inhibition of P-selectin functions significantly diminished platelet aggregation ($p<0.03$) and platelet-neutrophil adhesion *in vitro* ($p<0.01$) as well as platelet aggregate sizes under flow ($p<0.03$). Established aggregates were degraded, either via detachment of single platelets following addition of efomycine M, or via detachment of multicellular clumps when P-selectin-directed Fab-fragments were used. *In vivo*, selectin inhibition resulted in a greater than 50% reduction of platelet rolling in cutaneous venules ($p<0.01$), producing rolling fractions similar to those observed in P-selectin-deficient mice ($p<0.05$). Moreover, inhibition of selectin functions significantly decreased the thrombus size in $FeCl_3$-induced arterial thrombosis in mice ($p<0.05$). In an ischemia/reperfusion injury model in rats, small-molecule-mediated selectin inhibition significantly reduced myocardial infarct size from 18.9% to 9.42% ($p<0.001$) and reperfusion injury ($p<0.001$).

Interpretation and Conclusions

Inhibition of P-selectin functions reduces platelet aggregation and can alleviate platelet-related disorders in disease-relevant preclinical settings.

Key words: platelets, selectins, aggregation and thrombus formation.

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Platelet aggregation plays a major role in hemostasis.[1,2] However, platelet aggregation might also contribute to common pathological conditions including thrombosis, arterial occlusion in myocardial infarction and stroke. Indeed, platelet-related disorders are among the leading causes of death in industrialized countries.[3,4] Several macromolecular components provide a substrate for platelet adhesion, of which fibrillar collagen is considered to be the most thrombogenic compound of the vascular endothelium, supporting platelet adhesion as well as activation.[5,6] The interaction cascade between platelets and collagen involves consecutive steps, with a first phase of adhesion, followed by activation and a second phase of adhesion, secretion, and ultimately aggregation.[7,8] A number or receptors and signaling pathways, including P-selectin, have been implicated in this complex sequence of events, but their exact interactions are partly elusive. P-selectin is a C-type lectin that is expressed in α-granules of resting platelets and is translocated to the cell surface upon activation.[9] The role of P-selectin in thrombotic processes has long been discussed. Previous studies have suggested that P-selectin expression on platelets (it is the only selectin expressed by platelets) may influence the size and stability of thrombi.[10-12] Based upon comparisons between P-selectin-deficient and wild-type mice, it has recently been suggested that P-selectin expressed on activated platelets contributes to myocardial reperfusion injury.[13] However, the exact role of P-selectin in these processes is not clear, and its use as a therapeutic target structure has not been established. P-selectin expressed by endothelial cells is rapidly translocated to the cell membrane upon activation and may also contribute to aggregate formation.[14] Several types of cells, including leukocytes, endothelial cells and platelets, carry P-selectin ligands.[15] Leukocytes are thought to interact with activated platelets and thereby promote thrombosis and vascular occlusion, impairing the blood flow and exacerbating ischemia. Disruption of this binding was shown to be effective in reducing the incidence of re-occlusion in animal models of vascular injury.[11] Moreover, cell-derived microparticles carrying P-selectin glycoprotein ligand-1 (PSGL-1) are thought to circulate in the blood and accumulate in developing platelet-rich thrombi following vessel wall injury, thereby concentrating tissue factor at the site of vascular injury and initiating blood coagulation.[16] Thus, interfering with P-selectin functions might be a means to decrease thrombus formation in some cardiovascular disorders. However, based on partly contradictory results of previous studies,[17,18] the exact roles of P-selectin in platelet aggregation and in platelet-related cardiovascular disorders are still unclear.

Using P-selectin-specific antibodies, their Fab-fragments or efomycine M, a small-molecule compound that inhibits selectin functions,[19] we assessed the role of P-selectin in platelet aggregation *in vitro* as well as in platelet rolling, thrombus formation and myocardial infarction and reperfusion injury *in vivo*.

Design and Methods

Monoclonal antibodies

The P-selectin-specific function-blocking antibodies used were CLB-Thromb/6 (Immunotech, Marseille, France) and AK-4 (BD-PharMingen, Heidelberg, Germany). For functional studies, antibodies were purified by affinity chromatography from the hybridoma cell line WAPS12.2 (ATCC, Manassas, USA). Fab-fragments were generated using the ImmunoPure®-Fab-Kit (Pierce, Rockford, USA).

Platelet isolation

Whole blood from healthy individuals was diluted 4:1 in citrate buffer (pH 6.5) and centrifuged for 20 minutes at 280 g. The resulting platelet-rich plasma (PRP) was then centrifuged for 10 minutes at 500 g, and all supernatant was carefully removed. Platelets were then resuspended in 1/20th of the original volume in Tyrode's buffer (0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 0.5 M HEPES, 10% bovine serum albumin (BSA) and 10% glucose in H_2O, pH 7.35), hereafter referred to as platelet-rich cell-suspension.

Sialyl Lewis^x (sLe^x)-induced platelet aggregation

To study the role of P-selectin in platelet aggregation, 50 μL of platelet-rich cell-suspension were mixed with 20 μL of 1 M $CaCl_2$, 50 μL thrombin (1 U/mL) and 25 μL of 0.5 mg/mL biotinylated polyacrylamide coated with the selectin ligand, sLe^X (Lectinity, Moscow, Russia), in water in the presence or absence of 0.1 mg/mL P-selectin-specific antibodies or efomycine M (99.9% pure).[19] Platelets were incubated at room temperature for 30 minutes while gently shaken and, thereafter, fixed in 1% paraformaldehyde. Platelet aggregation was analyzed microscopically and quantitated digitally using ImageJ.[20]

In vitro adhesion assays

Platelet-neutrophil adhesion

Human platelets and neutrophils were freshly isolated by density gradient centrifugation.[21] After incubation with thrombin (0.25 U/mL, Sigma, Diesenhofen, Germany) for 10 minutes, the platelets were washed and incubated with 10^{-7} to 10^{-4} M of efomycine M for 10 minutes. Platelets were then mixed with neutrophils at a ratio of 10:1 in 96-well plates in 100 μL of culture medium (M199, Gibco/BRL, Karlsruhe, Germany). After 20 minutes, neutrophils with two or more adherent platelets (positive) and one or no adherent platelets (negative) were counted microscopically. Maximum binding was monitored in the absence of inhibitors (negative control); minimum binding was monitored in the presence of 5 mM EDTA to demonstrate calcium-dependence of the interaction (positive control).

Adhesion of neutrophils to srP-selectin

Neutrophils were intravitally labeled with ^{35}[S]-methionine (Amersham, Freiberg, Germany; 0.2 mCi/10^7 cells) for 3 h at 37°C.[19] Each well in a 96-well microtiter plate was coated with 50 µL of srP-selectin (Serotec/Biozol, Eching, Germany; 10 µg/mL) in TBS/$CaCl_2$, and blocked with 1% BSA. ^{35}[S]-methionine-labeled neutrophils (2×10^5/well) were then added to 100 µL of culture medium (RPMI1640, 10% fetal calf serum, 1 mM $CaCl_2$) containing efomycine M (10^{-7} to 10^{-4} M), the P-selectin-specific antibody (2 µg/well), or a mixture of both. The plates were then incubated for 1 h at 37°C, washed gently with TBS/$CaCl_2$, and bound cells were lyzed using 2% sodium dodecylsulfate. Bound radioactivity was quantitated by scintillation counting. Experiments were performed in sextuplicate.

Aggregometry

Light transmission was measured in platelet-rich plasma (160 µL) stimulated with collagen (10 µg/mL) in the presence or absence of efomycine M (0 to 0.3 mM), P-selectin-specific antibodies (6.5 µg) or Fab-fragments of the latter (6.5 µg). Transmission was recorded on a Fibrintimer 4-channel-aggregometer (APACT-Laborgeräte, Hamburg, Germany) for 10 minutes, and was expressed as relative units using plasma to determine 100% aggregation. Aggregation experiments were performed in duplicate for each sample from three donors.

Platelet aggregation under flow conditions

Heparinized human blood from three donors was diluted 1:1 in Hank's balanced salt solution (HBSS)/$CaCl_2$ with or without P-selectin-specific antibodies or efomycine M (0.01 to 0.3 mM). Cover slips (24×60 mm) were coated with 50 µL collagen (Kollagenreagenz Horm, Nycomed, Linz, Austria) at 0.5 mg/mL, dried at 37°C, and then incubated for 1 hour with 0.5% BSA. Transparent flow chambers with a slit depth of 50 µm and a slit width of 500 µm, equipped with the cover slips, were rinsed with HBSS/$CaCl_2$ and connected to a syringe containing the pre-incubated blood. Perfusion was performed using a pulse-free pump at a wall shear rate of 1000 s^{-1}. Microscopic phase-contrast images were recorded in real time. After 10 minutes of perfusion, non-aggregated cells were washed away using HBSS/$CaCl_2$. Images were analyzed off-line using MetaView Imaging software (Universal, Downington, USA). The number and size of aggregates were analyzed using ImageJ.[20]

Degradation of platelet aggregates under flow

Flow chamber experiments were performed as described above, with the difference that 1 mL of whole blood from three donors was perfused to allow the formation of stable aggregates, followed by perfusion with 500 µL of HBSS/$CaCl_2$. Aggregates were monitored microscopically in real time, and the chambers were perfused with 1.5 mL of HBSS/$CaCl_2$ containing efomycine M (0 to 0.3 mM), P-selectin-specific antibodies or Fab-fragments thereof (both at 1 mg/mL). Aggregate degradation was recorded in real-time for 3 minutes. Still images were taken at the beginning and end of the observation period and analyzed using ImageJ.

Platelet preparation for intravital microscopy

Heparinized blood from adult donor mice injected intraperitoneally with efomycine M (5 mg/kg in 1% dimethylsulfoxide, 5% Solutol, 5% ethanol in PBS) or vehicle 1 hour before bleeding was centrifuged at 250 g for 10 minutes, and platelet-rich plasma was gently transferred to a fresh tube. Platelets were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Invitrogen, Karlsruhe, Germany) and adjusted to a final concentration of 200×10^6 platelets/ 250 µL.[22]

Intravital microscopy

All animal experiments were approved by the local authorities. Intravital microscopy of mouse ears was performed as described elsewhere.[23,24] Briefly, wild-type- and P-selectin-deficient mice[23] (Jackson Laboratories, Bar Harbor, USA), both backcrossed for more than ten generations on a C57BL6 background, were anesthetized intraperitoneally using ketamin/rompun, and placed on a homeothermic blanket. A catheter was placed micro-surgically into the right carotid artery for injection of CFDA-SE-labeled platelets, efomycine M (4 mg/kg) or P-selectin-specific antibodies (4 mg/kg). The left ear was gently placed on a microscope slide and covered with glycerin and a cover slip. The cutaneous microcirculation was continuously recorded using a 1/3" DSP 3-CCD camera (Sony, Köln, Germany). Cell behavior in individual vessels was analyzed off-line. Cells were considered non-interacting when they moved at the velocity of the blood flow (V_{free}), whereas lower velocities were defined as rolling. A total of 11 vessels in four wild-type mice and six vessels in three P-selectin-deficient mice treated with efomycine M as well as six vessels in three wild-type animals treated with P-selectin-specific antibodies were analyzed before and after the respective treatments.

$FeCl_3$-induced arterial thrombosis

Four to five-week-old C57BL6 mice were injected intraperitoneally with 5 mg/kg efomycine M (n=18 mice) or vehicle (n=16 mice) 1 hour before they were anesthetized intraperitoneally. One hundred million CFDA-SE-labeled platelets/mouse were injected through the tail vein; efomycine M-treated mice received platelets from efomycine M-treated donors and *vice versa*. The mesentery was externalized through an abdominal incision. Arterioles of 35-60-µm in diameter were visualized microscopically using a CCD camera (Visitron, Puchheim, Germany). After topical application of a filter paper saturated with 20% $FeCl_3$ for 1 minute, arterioles were monitored for 40 minutes or until complete occlu-

sion occurred.[25] Thrombus formation was analyzed using ImageJ.

Tail bleeding time experiments

Wild-type or P-selectin-deficient C57BL6 mice (four mice/group) were injected intraperitoneally with 5 mg/kg efomycine M resulting in serum concentrations of >0.1 mM after 1 h, i.e. concentrations sufficient to inhibit P-selectin and platelets *in vitro*.[19] Mice that received vehicle only served as controls. Ninety minutes after injection, the mice were placed in a restrainer and the distal 5 mm of tail was amputated using a scalpel. The tail was then blotted with filter paper every 10 seconds until the paper was no longer blood-stained.[26]

Myocardial infarction and reperfusion injury

One hour after intraperitoneal injection of efomycine M (0; 0.03; 0.1; 1.0 mg/kg; n=10 animals/group), Lewis rats were anesthetized intraperitoneally with thiopental. For substance administration and blood pressure recording, the left jugular vein and the right carotid artery, respectively, were catheterized with a polyethylene tube. The animals were ventilated through a tracheal tube.

Following thoracotomy, a thin thread (Prolene®, 5-0) was placed microsurgically around the left anterior descending artery with a stitch through the myocardium. Tightening the loop occluded the artery and induced ST-segment elevation on the electrocardiograph. The loop was loosened in order to perfuse the ischemic myocardium. Sham operated animals (negative controls) were treated identically, but the loop was not tightened. After 30 minutes of occlusion and 90 minutes of reperfusion, the heart was removed and perfused via the aorta with TTC-solution (2,3,5-triphenyltetrazolium-chloride 1.5% in a 1:1 mixture of water and PBS) for 15 minutes to differentiate between viable and irreversibly injured myocardium.[27,28] Viable myocardium (bright red) was separated surgically from infarcted tissue (unstained). The infarct size was expressed as a percentage of the weight of the ventricles.

Myeloperoxidase assay

Myeloperoxidase (MPO) is widely used as a marker enzyme for granulocytes.[29-32] Briefly, frozen myocardial samples were immersed in liquid nitrogen, pulverized and homogenized in a N-acetyl-N,N,N-trimethylammonium-bromide-solution (0.5% in phosphate buffer, pH 7.4). Following three cycles of thawing and freezing in liquid nitrogen, the samples were centrifuged for 20 minutes at 4000 rpm. The volume of the supernatant was measured and four samples of 30 µL each were pipetted onto a microtiter plate. The reaction was started with 270 µL of substrate solution (250 µL phosphate buffer, 50 mM, pH 6.0, 10 µL H_2O_2, 10 µL o-dianisidine solution [0.6% in DMSO]), and the extinction was measured over 3 minutes. ΔE/min was calculated as $(E_{t=3min}–E_{t=0})/3$. The MPO concentration was calculated as: (ΔE/min • 1000 • volume [mL])/(8.3 • weight (g)) = MPO (nmol/min • g^{-1} fresh weight)

Statistical analysis

Data are displayed as mean (±SD or SEM as indicated); *p* values were determined using the two-tailed t-test, and *p* values <0.05 (confidence interval of 95%) were considered statistically significant. All statistical tests were two-sided.

Results

Antibody- or small-molecule-mediated inhibition of P-selectin functions diminishes platelet aggregation in vitro

In order to assess the activity of selectin-directed inhibitors on platelet aggregation, thrombin-activated human platelets were incubated with multimeric sLe^x. In addition, P-selectin-specific antibodies or efomycine M were added to the platelet suspension. As expected, platelet aggregation occurred as a result of activation with low-dose thrombin (Figure 1A, first two panels). However, the aggregate size was dramatically increased when sLe^x was added (Figure 1A, third panel, p=0.008 as compared to thrombin alone). The latter effect was completely abolished in the presence of P-selectin-specific antibodies or efomycine M (Figure 1A, last two panels and Figure 1B, p<0.001). These data show that P-selectin plays an important role in platelet aggregation *in vitro* and that P-selectin-specific antibodies or the small-molecule inhibitor, efomycine M, inhibits platelet aggregation. To confirm the effect of P-selectin inhibition on platelet aggregation with a second, independent method, standardized aggregometer experiments were performed. Platelets were isolated and activated with collagen, a potent inducer of thrombus formation.[7,8] This incubation was performed in the presence or absence of efomycine M (0 to 0.3 mM), P-selectin-specific antibodies or the Fab-fragments of these antibodies (Figure 2). Again, it was found that efomycine M significantly inhibited platelet aggregation in a dose-dependent fashion (Figure 2A and B). Surprisingly, the use of whole P-selectin-specific antibodies did not decrease aggregate formation in this system (Figure 2A, C). In order to exclude that the failure of P-selectin-directed IgG to inhibit aggregation was due to potential cross-linking of activated platelets by the intact bivalent IgG molecule, we generated Fab-fragments of this antibody. These fragments significantly reduced the maximal aggregation from 71.3 (±6.4%) in the control samples to 47.0 (±6.7%) in the Fab-fragment-treated platelet suspensions (p<0.03 as compared to controls, Figure 2C). Thus, this system again showed P-selectin-dependent platelet aggregation. Complex pathophysiological processes, such as reperfusion injury

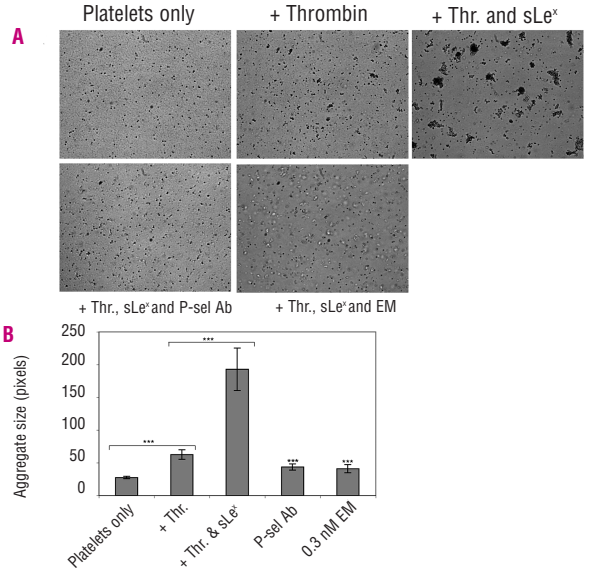

Figure 1. P-selectin-dependent static platelet aggregation *in vitro*. A. The interaction of thrombin-activated platelets with multimeric sialylated Lewis^x was analyzed in a static assay. Isolated platelets were incubated in the absence (first panel) or presence (second to fifth levels) of thrombin and calcium. Sialylated Lewis^x was added to the platelets (third to fifth panels) and the induced aggregate formation was inhibited using P-selectin-specific antibodies (fourth panel) or efomycine M (fifth panel). Microscopic images were taken at a 40 x magnification. The figure shows a representative example of three independent experiments, performed with platelets from different donors, which all showed similar results. B. Digital analysis revealed a significant increase of platelet aggregate size following addition of sialylated Lewis^x; this selectin-dependent aggregation was completely abrogated in the presence of P-selectin-specific antibodies or efomycine M.

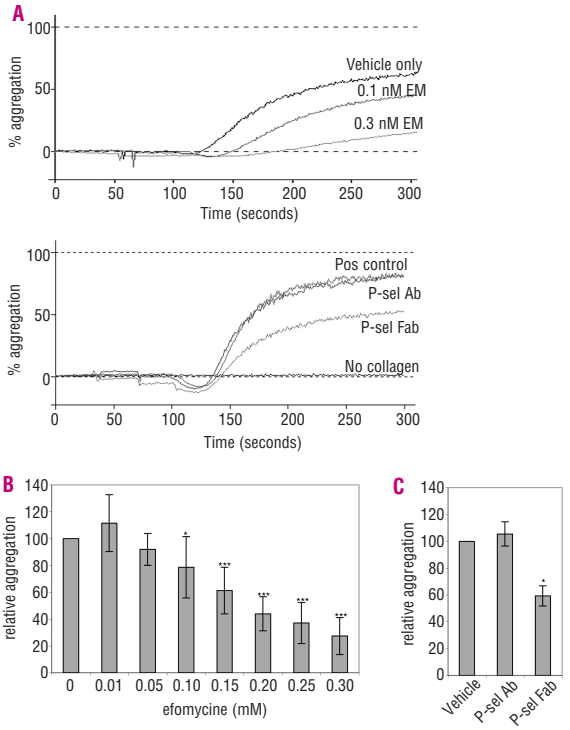

Figure 2. Aggregometry shows P-selectin-dependent aggregation of platelets. A. Platelet aggregation was assessed by aggregometry in the presence or absence of efomycine M (EM, upper panel), P-selectin-specific antibodies or Fab-fragments of these antibodies (lower panel). B. Reduction of platelet aggregation in the presence of efomycine M at the indicated concentrations. C. Inhibition of platelet aggregation when Fab-fragments of P-selectin-specific antibodies were used (third column), whereas P-selectin-specific IgG did not have an inhibitory effect (second column) as compared to controls (first column). The percentage aggregation in Figures 2B and C was calculated relative to the aggregation after 500 s incubation at 37°C under continuous stirring in platelet-poor plasma (0% aggregation) and platelet-rich plasma (100% aggregation) treated with vehicle only. The data are presented as mean ±SD of three independent experiments in which each data point was analyzed in duplicate.

following myocardial infarction, also involve platelet adhesion to different tissue structures and other selectin-mediated interactions, such as influx of inflammatory cells. To approach at least some aspects of such complex situations *in vitro*, adhesion assays were performed evaluating the P-selectin-dependent binding of freshly isolated thrombin-activated human platelets, which translocate P-selectin to the plasma membrane, with neutrophilic granulocytes, which express glycosylated P-selectin ligands.[33] Adhesive interactions between activated platelets and neutrophils are mediated almost exclusively by calcium-dependent binding of P-selectin to its carbohydrate-containing ligands.[34] Platelet-neutrophil adhesion was assessed by quantitating clusters formed by neutrophils and platelets in the presence of P-selectin-specific antibodies or efomycine M. It was found that adhesion of neutrophils to activated platelets was significantly reduced in a concentration-dependent fashion in the presence of the antibody- or efomycine M-containing mixture ($p<0.01$ in both cases as compared to vehicle-treated controls, Figure 3A). In another series of experiments, isolated human neutrophils were incubated with either culture medium or fixed with 4% paraformaldehyde for 10 minutes prior to the adhesion assay. Since binding of activated platelets was comparable and efomycine M similarly inhibited neutrophil/platelet adhesive interactions in both cases ($p<0.001$ as compared to controls, Figure 3B), the adhesion-blocking effect of efomycine M was not due to non-specific or toxic events. Moreover, identical anti-adhesive effects were observed when efomycine M was added to the platelets either before or after activation with thrombin. Thus, efomycine M did not affect the activation of platelets (*data not shown*). The hypothesis of a P-selectin-directed anti-adhesive action of efomycine M was supported by similar results obtained with other inhibitors specifically interfering with selectin functions, such as sulfatides, the sulfated polysaccharide fucoidin, and peptide fragments of the lectin-like domain of P-selectin (*data not shown*). To formally confirm that it is the P-selectin-mediated adhesion of leukocytes that can be inhibited by efomycine M, srP-selectin was coated onto microtiter plates and ^{35}[S]-labeled neutrophils were allowed to adhere to this matrix in the presence of normal culture medium with or without efomycine M at different concentrations or with a P-selectin-blocking

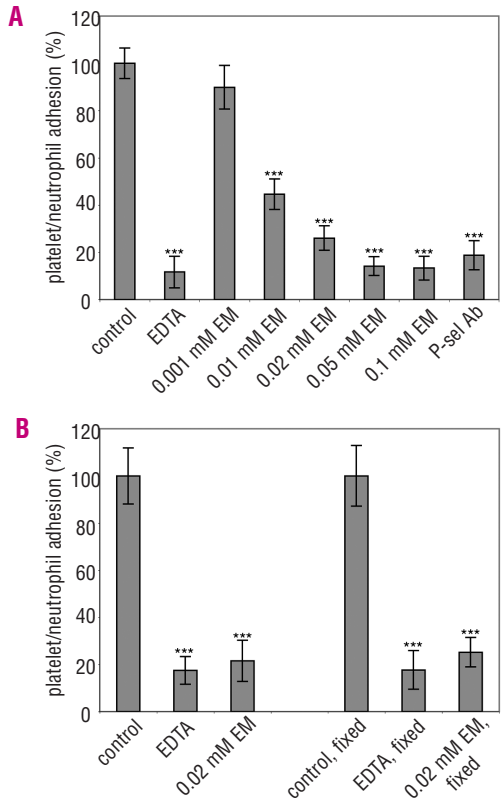

Figure 3. A. Neutrophils and platelets were isolated from human blood, platelets were activated by thrombin, and adhesion assays were performed, as outlined in the *Material and Methods*, in normal culture medium (first column), in the presence of 5 mM EDTA to chelate divalent cations (second column), in the presence of a P-selectin-blocking antibody or in the presence of efomycine M at different concentrations as indicated. The experiment shown is representative of three experiments that gave similar results. **B.** Neutrophil-platelet adhesion assays were performed as described above. The results shown in the right panel were for neutrophils fixed with 4% paraformaldehyde prior to the adhesion assays. Adhesion assays were performed in normal culture medium (left columns, in the presence of 5 mM EDTA (middle columns), or in the presence of of 2×10^{-5} M efomycine M (right columns). The experiment shown is representative of three independent experiments that gave similar results.

antibody. As compared to the controls, both efomycine M and the antibody showed a significant inhibitory effect on P-selectin binding of leukocytes (*data not shown*).

Interference with P-selectin functions diminishes platelet aggregation under flow

To assess P-selectin functions on platelets under dynamic conditions, platelet aggregate formation on collagen was analyzed using dynamic video microscopy to mimic the circumstances in a blood vessel in which thrombus formation occurs under flow conditions and shear stress. Human blood was diluted 2-fold in HBSS with 2 mM $CaCl_2$ only, or in HBSS/$CaCl_2$ containing P-selectin-specific antibodies or efomycine M. It was found that numerous aggregates began to form on the collagen matrix under all conditions. However, the size of the individual aggregates was moderately, but significantly, reduced in a concentration-dependent manner when using efomycine M ($p<0.03$ as compared to controls). This effect was even stronger when P-selectin-specific antibodies were used ($p<0.01$ as compared to controls). Thus, it appeared that the initial contact of platelets with collagen activated the platelets and stimulated aggregate formation, while P-selectin inhibition impaired the growth of the platelet aggregates under dynamic flow conditions (*data not shown*).

Considering the course and consequences of platelet-related cardiovascular disorders, it appears to be clinically perhaps more important to reduce already existing aggregates than to prevent the development of new ones. Therefore, we assessed the potential of P-selectin inhibitors to degrade already existing aggregates in a dynamic system under conditions of shear flow, whereby platelet aggregation on collagen took place first, followed by rinsing with medium containing efomycine M, P-selectin-specific IgG antibodies or Fab-fragments of these antibodies. The aggregates were monitored by real-time video microscopy for the complete duration of the experiment, and aggregate sizes were quantitated digitally using an objective, morphometric algorithm. As compared to medium or vehicle alone, efomycine M, P-selectin-specific antibodies or Fab-fragments of these antibodies significantly reduced the aggregate size by >60% (Figure 4, $p<0.001$ comparing aggregate size before treatment with aggregate size after 9 minutes of treatment). Interestingly, Fab-fragments again had a significantly more pronounced effect than had the whole antibody, which is consistent with the results of our abovementioned aggregation experiments (Figure 2).

Of note, when the dynamics of the size reduction of platelet aggregates through selectin-inhibition was assessed in more detail, an interesting difference between P-selectin-directed antibody fragments and the small-molecule selectin inhibitor, efomycine M, became apparent: while antibody-treatment resulted in the detachment of large multicellular clumps of platelets from the aggregates, the lytic effect of efomycine M occurred via detachment of very small groups or even single platelets from the aggregates (*examples shown in supplementary videos s1-s3*). The molecular basis of this conspicuous difference remains to be determined. In any case, considering that the detachment of large fragments from thrombi *in vivo* may result in embolization with potentially dangerous consequences, a gentle reduction of platelet aggregate size might bear some advantage.

Rolling of platelets on endothelial cells is reduced by inhibition of P-selectin functions

Platelet rolling along the endothelial wall is a prerequisite for firm attachment and subsequent thrombus formation in intact vessels. In addition, vascular endothelial cells also express selectins, which may be involved in platelet aggregation.[35] Therefore, complementary *in vivo* experiments were performed in which the effects of P-

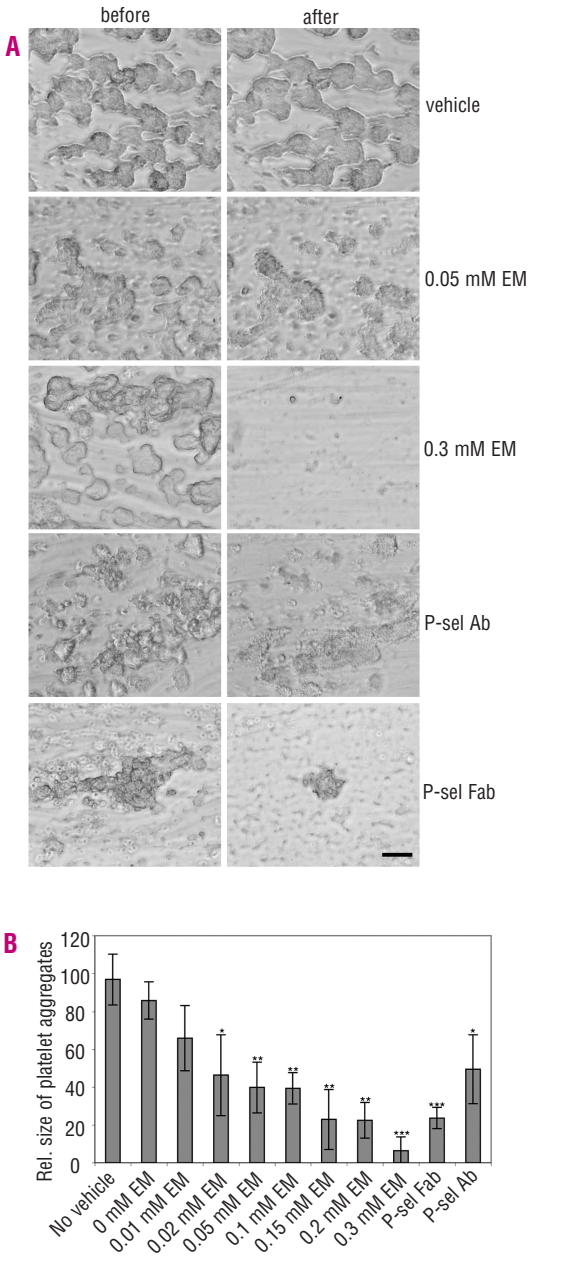

Figure 4. A. P-selectin-specific antibodies, Fab-fragments of these antibodies or efomycine M at different concentrations (dissolved in HBSS + 2 mM $CaCl_2$), were perfused for 20 minutes at a wall shear rate of 1000 s^{-1} over already formed platelet aggregates as indicated. Images were recorded before and after perfusion (the bar in the bottom right corner equals 10 µm length). B. The aggregate sizes were calculated using ImageJ-software. Digital quantitation of aggregate size revealed that the aggregates could be degraded significantly when P-selectin-specific antibodies, Fab-fragments or efomycine M were used. The data represent the mean ± SD of three independent experiments. This figure corresponds to supplemental videos 1-3.

selectin-specific antibodies and efomycine M on the interaction of platelets with post-capillary venules in murine skin were assessed using intravital microscopy in wild-type mice as well as in P-selectin-deficient mice. In untreated wild-type mice, 25.4% (±14.7%) of the platelets showed a rolling motion along the endothelium (Figure 5A, left column and *supplementary video s4*). When the animals were injected with efomycine M (5 mg/kg), platelet rolling decreased significantly by 67.5% ($p<0.01$, Figure 5A, *supplementary video s5*), and injection of P-selectin-specific antibodies caused a 47.7% reduction of platelet rolling ($p=0.02$ as compared to vehicle-treated controls (Figure 5B). Platelet-rolling in P-selectin-deficient mice was comparable to that observed in efomycine M-treated wild-type mice ($p<0.05$ compared to wild-type mice) and could not be further reduced by injection with efomycine M ($p=0.03$ compared to wild-type mice, Figure 5A). These results demonstrate that the small-molecule inhibitor had a similar effect *in vivo* when compared to P-selectin-specific antibodies or to P-selectin deficiency. Bleeding time experiments showed that treatment of both wild-type and P-selectin-deficient mice with efomycine M did not result in prolongation of tail bleeding (*data not shown*) indicating that hemostasis following injury was not affected by efomycine M, at least not in the model tested here.

Thrombus formation in vivo is impaired by selectin inhibition

To assess the effect of selectin inhibition on thrombus formation in a disease-relevant situation *in vivo*, oxidative injury was induced in mesenteric arterioles and thrombus formation was examined by *in vivo* fluorescence microscopy. C57BL6 mice (4 to 5-weeks old) were injected intraperitoneally with vehicle (n=16 mice) or 5 mg/kg efomycine M (n=18 mice) 1 hour before they received an intravenous injection of 10^8 fluorescently labeled platelets isolated from syngeneic donors that had also been treated with efomycine M. Surgically exposed mesenteric arterioles were injuried by topical application of $FeCl_3$, which induces the formation of free radicals, leading to disruption of the vascular endothelium.[36] Following injury, thrombus formation was monitored by real-time *in vivo* fluorescence microscopy, whereby still images were recorded every minute. The onset of thrombus formation (defined as the first time point at which a thrombus >20 µm was observed) and the changes in size of the thrombi over time were recorded and analyzed digitally (Figure 6A). A small, but consistent delay in the onset of thrombus formation could be observed in the efomycine M-treated group as compared to in vehicle-treated mice (8.1±3.7 minutes and 10.7±6.3 minutes, respectively; Figure 6B). However, this difference did not reach statistical significance. In contrast, when thrombus development was analyzed, a significant difference in the average size of the thrombi formed was detected (Figure 6B, $p<0.05$ after 25 minutes). These results were consistent with our aforementioned flow chamber experiments that also showed no difference in the onset of platelet aggregate formation, but showed significant differences in aggregate size when P-selectin inhibition was compared with control conditions.

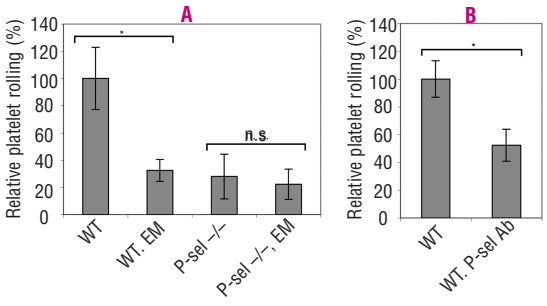

Figure 5. Rolling of activated platelets in murine post-capillary venules can be inhibited by small-molecule- or antibody-mediated blockage of P-selectin. A. Rolling of activated platelets from wild-type-mice (n=11) or P-selectin-deficient mice (n=6) in the absence or presence of efomycine M (5 mg/kg) was observed in murine skin post-capillary venules by intravital fluorescence microscopy as outlined in the *Materials and Methods*. B. The effect of P-selectin-specific antibodies on the rolling of activated platelets from wild-type-mice (n=6) was analyzed in a different set of experiments. Data are displayed as relative rolling fraction, which refers to the rolling of activated platelets (100%). Data are presented as mean ± SEM. This figure corresponds to supplemental videos 4-6.

Inhibition of selectin functions has a moderate effect on acute myocardial infarction and significantly alleviates reperfusion injury

The effect of efomycine M on ischemia/reperfusion injury was tested in a large series of experiments assessing cardiac hypoxia and reoxygenation in rats. Given that rats have little collateralization of the coronary arteries, they provide an ideal *in vivo* model in which the occlusion of a coronary artery induces reliable and reproducible ischemia. After occlusion of the left anterior descending artery for 30 minutes and reperfusion for 90 minutes, infarct size and concentration of MPO in the infarcted myocardium were determined. MPO was used as a reliable marker enzyme for granulocyte accumulation, since the adhesion of granulocytes is increased due to P-selectin expression on endothelium or platelets bound to the endothelium.[35] The infarct size was significantly reduced when animals were treated with efomycine M at 1 mg/ kg body weight 1 hour prior to the occlusion (untreated: 18.9±0.8% of ventricle weight compared to efomycine M-treated: 9.42±1.0% of ventricle weight, $p<0.001$, Figure 7A). Lower concentrations of efomycine M did not result in significant reduction of infarct size (Figure 7A). However, when MPO was assessed as a surrogate marker for reperfusion injury, a significant dose-dependent alleviation was observed even at lower concentrations of efomycine M (Figure 7B). Thus, small-molecule-mediated inhibition of selectin functions resulted in marked alleviation of reperfusion injury following myocardial infarction.

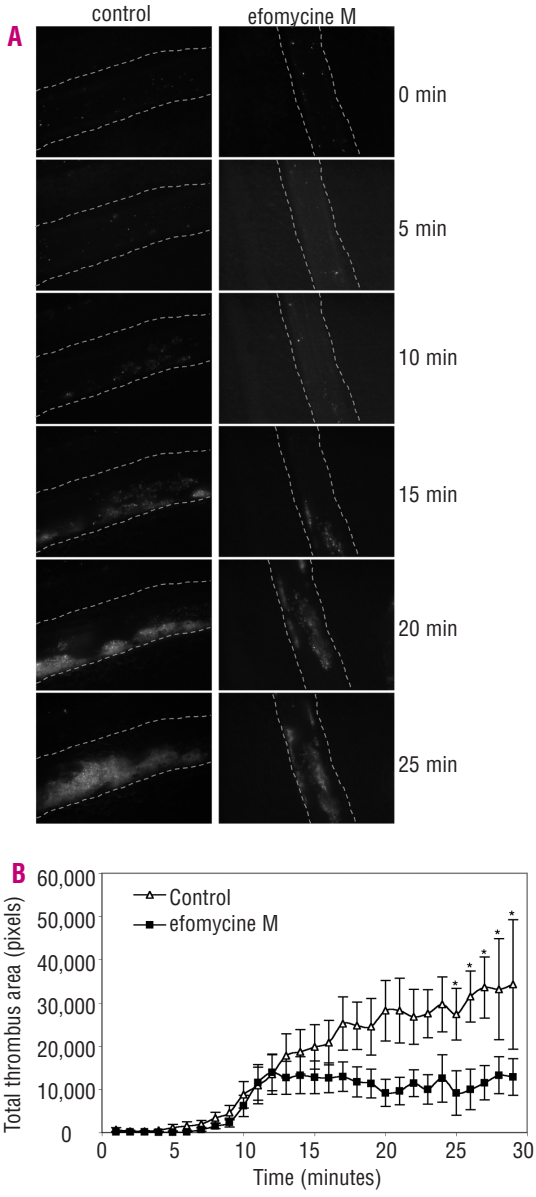

Figure 6. Thrombus sizes in $FeCl_3$-injured mesenteric vessels of mice can be reduced by blocking selectin functions. A. Thrombus formation *in vivo* was monitored in mesenteric arterioles after topical application of 20% $FeCl_3$ as outlined in the *Design and Methods*. Representative examples of the recorded images from an arteriole of a vehicle-treated mouse (left column of photomicrographs) and efomycine M-treated mouse (right) are shown. B. Digital quantitation of thrombus sizes revealed that the size of thrombi formed after $FeCl_3$ injury was significantly reduced in efomycine M-treated mice (indicated by *). Data are presented as mean ± SEM.

Discussion

Most, if not all, cardiovascular disorders, the leading causes of death in industrialized countries,[37] involve dysregulated platelet functions.[38] For example, it is thought that platelets are critically involved in coronary artery thrombosis, the predominant pathophysiologic mechanism underlying coronary occlusion and, consecutively, myocardial infarction.[38] Several lines of experimental evidence coupled with prospective cohort and case-control studies have long indicated that platelet-directed therapies could reduce the risk of thrombosis and myocardial infarction.[39,40] Hence, the identification of molecular mechanisms underlying dysregulated platelet functions could lead to selective therapeutic modulations. P-selectin (CD62P) appears to be one of the common

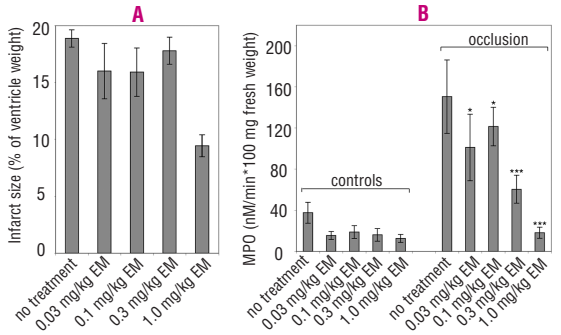

Figure 7. Selectin inhibition in a rat model of ischemia/reperfusion injury. **A.** Rats were injected intraperitoneally with efomycine M prior to a 30-minute occlusion of the coronary artery, followed by a 90-minute reperfusion period as detailed in the *Design and Methods*. The infarct size was measured relative to the ventricle weight, which was significantly reduced in rats treated with efomycine M at 1 mg/kg body weight. **B.** Myeloperoxidase values were measured as a marker of reperfusion injury and showed a significant reduction in rats treated with > 0.03 mg/ kg efomycine M as compared to the levels that were measured in the sham operated controls. The data are presented as the mean ± SD; ten animals were included in each group.

denominators in a number of complex platelet-related cardiovascular diseases,[11,13,17,18,41,42] thus providing a rationale for modulating P-selectin functions in order to treat and/or prevent such disorders. P-selectin expressed by activated endothelial cells may also contribute to disease processes. Thus, inhibition of P-selectin functions in inflammatory responses in general, and leukocyte rolling in particular is predicted to be useful, especially in the experimental setting of reperfusion injury. Previous studies have shown that the inhibition of E-, P-, or L-selectin functions alone might not result in the successful treatment of a disease and it is still not clear whether other adhesion molecules might compensate for the effect caused by blocking selectin functions. These limitations are exemplified by the unsatisfactory results of early clinical trials in which single selectin-directed compounds were tested in cardiovascular applications.[43,44] Nevertheless, selectin-directed therapies, especially those similtaneously targeting all three selectins, might be beneficial for the outcome of cardiovascular diseases. Formation of a platelet-rich thrombus on the vessel wall is a complex dynamic process that conceptually occurs in three distinct steps, platelet adhesion, followed by activation and secretion, and then aggregation.[38] Several aspects of this cascade can be modeled *in vitro* using a combination of static and dynamic assays, as in our study. Aggregate formation in all systems tested was dependent on P-selectin, as demonstrated by impaired clotting in the presence of efomycine M, P-selectin-directed antibodies or their Fab-fragments. Of note, P-selectin-specific IgG did not appear to have an inhibitory effect in aggregometry, using platelet-rich plasma, whereas efomycine M and Fab-fragments did show significant inhibitory effects. This finding was unexpected and is in apparent contrast to the results found in the flow chamber experiments. Some previous studies, in which the use of Fab-fragments was neglected, failed to demonstrate an anti-aggregation effect of P-selectin-specific IgG[18,42] which may have been due to cross-linking of P-selectin between platelets, thus antagonizing potential anti-adhesive effects. Another possible explanation for this apparent discrepancy is that the use of whole blood in the flow chamber assay promotes interactions between neutrophils/monocytes and platelets as has previously been described to occur in thrombus formation,[11] an interaction that cannot occur in the aggregometry experiments. PSGL-1 has been identified as one of the ligands for P-selectin on neutrophils and monocytes.[45,46] Even though PSGL-1 is also present on platelets, its expression is 25- to 100-fold lower on platelets as compared to on leukocytes.[47] Given that we have demonstrated that inhibition of P-selectin impairs platelet-platelet, platelet-leukocyte and platelet-endothelial cell adhesion, it is conceivable that this principle interferes with several steps in the pathophysiological cascade of thrombus formation. However, the relative contribution to each of these aspects remains to be determined. In any case, our results indicate that P-selectin-dependent platelet functions are critically involved in the formation and stabilization of platelet aggregates, thus suggesting that modulating P-selectin functions may be a valuable strategy to prevent and/or treat thrombus formation *in vivo*. The aggregation of platelets under flow conditions indicated that interfering with P-selectin decreases the stability and size of aggregates. Our data and those published by others[10,48] show that P-selectin plays an important role in thrombus stability and aggregate size *in vitro* and *in vivo*. Therefore, the use of small-molecular compounds that decrease stability and size of thrombi, but do not impair hemostasis, is of therapeutic interest. Interestingly, we found that efomycine M as well as P-selectin-directed Fab-fragments reduced the size of established aggregates,. Of note, when aggregate degradation was analyzed at high resolution, we observed size reduction via detachment of individual platelets rather than detachment of platelet aggregates when efomycine M was used, suggesting a low risk of embolization *in vivo*. In contrast, P-selectin-specific antibodies resulted in aggregate degradation via detachment of multicellular clumps (*supplementary videos s1-s3*). This novel observation might be of clinical relevance. However, the molecular basis of this interesting and potentially important difference is thus far unknown. P-selectin is an interesting target molecule since it is not only expressed on platelets, but also on activated endothelial cells, which are involved in cardiovascular diseases.[35] Rolling of platelets along the endothelial lining is a prerequisite for firm attachment in intact vessels and, therefore, for the formation of thrombi and atherosclerotic plaques. It is thought that platelet P-selectin is indispensable for interactions of activated platelets with artherosclerotic arteries and leukocytes/monocytes, whereby platelet rolling on the endothelium increases the influx of inflammatory cells to atherosclerotic lesions.[49] In our experiments,

platelet rolling within post-capillary venules could be decreased similarly by efomycine M and P-selectin-specific antibodies.[23] In our $FeCl_3$-model of arterial injury, endothelial cells played a minor role, if any, since most of them were removed by the experimental procedure.[50] Therefore, it can be assumed that the effect of efomycine M was due to inhibition of P-selectin on platelets. The onset of thrombus formation in $FeCl_3$-injured mesenteric vessels of efomycine-treated mice was slightly delayed, and a significant difference was seen in thrombus size between efomycine M-treated mice and untreated animals. Although the mechanism by which thrombus formation is triggered in this model is not entirely clear, the thrombi are morphologically similar to those found in human atherosclerosis.[51] Although rapid reperfusion of ischemic myocardium is essential for restoring normal heart functions, this return of perfusion can, apparently paradoxically, enhance the destruction of reversibly damaged cardiomyocytes. This reperfusion injury leads to the progression of myocardial dysfunction/infarction.[37] The pathogenesis of reperfusion injury is complex and not completely understood. Several cell types as well as an intertwined network of soluble mediators and adhesion molecules orchestrate this process.[41] However, given that several lines of evidence indicate that platelets play an important role in reperfusion injury,[13] modulating selectin-dependent functions of platelets and other cells may enhance myocardial salvage in patients with myocardial infarction.[52] Although all three selectins are thought to be involved in ischemia and reperfusion injury, P-selectin appears to play a major role since it is upregulated within minutes following a stimulatory event.[9] In the rat model of ischemia/reperfusion injury employed in our study, blocking selectin functions had a beneficial effect on infarct size and reperfusion injury. In complex *in vivo* models, such as the one used here, it is generally difficult to assess the relative contribution of a given cell type and/or molecule to the overall pathogenesis. This study provides comprehensive experimental evidence that P-selectin-dependent functions of platelets and other cells play a role in myocardial infarction and that targeting these functions by using a novel small-molecule compound could have therapeutic potential for the treatment of diseases in which platelets play a role.

Authors' Contributions

GJO has designed and performed research, analyzed data (flow chamber, in vitro-aggregation, adhesion, tail bleeding) and wrote the manuscript; MP has performed research and analyzed data (in vivo thrombus formation in the FeCl3-model); RJL performed research and analyzed data (intravital microscopy and platelet rolling); TK performed research and analyzed data (myocardial infarction and reperfusion injury); WHB performed research and analyzed data (intravital microscopy); BN performed research and analyzed data (in vivo thrombus formation); MPS designed the study, performed research, analyzed data (several in vitro experiments, intravital microscopy, thrombus formation) and wrote the mauscript.

All authors critically revised the manuscript and approved the final version.

Conflict of Interest

The authors reported no potential conflicts of interest.

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