The Novel Oral Syk Inhibitor, BL1002494, Protects Mice From Arterial Thrombosis and Thromboinflammatory Brain Infarction

Judith M.M. van Eeuwijk,* David Stegner,* David J. Lamb, Peter Kraft, Sarah Beck, Ina Thielmann, Friedemann Kiefer, Barbara Walzog, Guido Stoll, Bernhard Nieswandt

Objective—Ischemic stroke, which is mainly caused by thromboembolic occlusion of brain arteries, is the second leading cause of death and disability worldwide with limited treatment options. The platelet collagen receptor glycoprotein VI (GPVI) is a key player in arterial thrombosis and a critical determinant of stroke outcome, making its signaling pathway an attractive target for pharmacological intervention. The spleen tyrosine kinase (Syk) is an essential signaling mediator downstream of not only GPVI but also other platelet and immune cell receptors. We sought to assess whether Syk might be an effective antithrombotic target.

Approach and Results—We demonstrate that mice lacking Syk in platelets specifically are protected from arterial thrombus formation and ischemic stroke but display unaltered hemostasis. Furthermore, we show that mice treated with the novel, selective, and orally bioavailable Syk inhibitor BL1002494 were protected in a model of arterial thrombosis and had smaller infarct sizes and a significantly better neurological outcome 24 hours after transient middle cerebral artery occlusion, also when BL1002494 was administered therapeutically, that is, after ischemia.

Conclusions—These results provide direct evidence that pharmacological Syk inhibition might provide a safe therapeutic strategy to prevent arterial thrombosis and to limit infarct progression in acute stroke. (Arterioscler Thromb Vasc Biol. 2016;36:1247-1253. DOI: 10.1161/ATVBAHA.115.306883.)

Key Words: mice ■ platelets ■ stroke ■ Syk kinase ■ thrombosis

Platelet aggregation is not only essential for hemostasis but also a major pathomechanism underlying myocardial infarction and ischemic stroke. Therefore, platelet inhibition is commonly used in secondary stroke prevention.1 In acute stroke, however, revascularization by recombinant tissue-type plasminogen activator or mechanical thrombectomy are the only therapeutic means1,2 with limited efficacy.3 Addition of plasminogen activator or mechanical thrombectomy are the only therapeutic means1,2 with limited efficacy.3 Addition of conventional antiplatelet agents, such as acetylsalicylic acid, led to bleeding complications.4 To improve functional outcome in acute stroke, novel antithrombotic strategies preserving physiological hemostasis are eagerly awaited.

See accompanying editorial on page 1054

The spleen tyrosine kinase (Syk) is a 72-kDa multi-domain cytoplasmic tyrosine kinase, which is highly expressed in hematopoietic cells. Syk acts downstream of immunoreceptor tyrosine-based activation motif (ITAM)–coupled receptors, such as the B-cell receptor or Fcγ receptors (FcγRs) in macrophages. In platelets, Syk is essential for signaling downstream of the (hem)ITAM receptors glycoprotein VI (GPVI), C-type lectin-like receptor 2 (CLEC-2), and the FcγRIIA, the latter being present on human but not on murine platelets.5 FcγRIIA signals through an ITAM in its cytoplasmic tail and is critically involved in immune-mediated thrombocytopenia and thrombosis, but it also enhances platelet integrin outside-in signaling via Syk.5 GPVI signals via the noncovalently associated ITAM-bearing Fcγγ-chain. On ligand-induced receptor ligation of GPVI, the tyrosine residues in the ITAM become phosphorylated by Src family kinases, inducing the recruitment and activation of the tandem Src homology 2 domain–containing kinase Syk. CLEC-2 only contains 1 copy of the YxxL motif (hemITAM) and is expressed as a homodimer on the platelet surface. In contrast to classical ITAM signaling, recent studies suggest that Syk itself phosphorylates the hemITAM within CLEC-2,6 whereas the Src family kinases together with Syk are involved in the
regulation of the downstream signaling cascade via the linker of activated T cells signalosome, ultimately leading to the activation of phospholipase Cγ2. Because of the central role of Syk in immunologic processes, several Syk inhibitors have been developed and clinically used, such as the prodrug fostamatinib (R788)7,8 and its active metabolite R406.9 However, these inhibitors have a rather limited specificity and adverse effects.10 Recently, entospletinib (GS-9973) was developed and described as a more selective and orally efficacious Syk inhibitor11; however, Thoma et al12 showed only modest activity of GS-9973. Phase II trials are now underway to assess its effectiveness in treating leukemia.13 Syk inhibitors have been used in the treatment of allergy,14 B-cell malignancies,7 heparin-induced thrombocytopenia,15 and autoimmune diseases, such as rheumatoid arthritis and immune thrombocytopenic purpura.16 Although Syk is critical for platelet signaling, a possible significance of Syk-mediated signaling in the pathogenesis of ischemic stroke has not been established to date. Here, we show that a platelet-specific Syk deficiency or treatment with the novel selective and orally bioavailable Syk inhibitor, BI1002494, profoundly protects mice from arterial thrombosis and cerebral infarct progression while not increasing the risk of intracranial hemorrhage.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Sykfl/fl, Pf4-Cre−/− mice displayed normal basal blood parameters and surface expression of major platelet glycoproteins (Table and not shown). Flow cytometric analyses revealed unaltered platelet integrin activation (JON-A/PE binding) and degranulation (P-selectin exposure) in response to G-protein–coupled agonists but abolished responses to GPVI and CLEC-2 agonists collagen-related peptide and rhodocytin, respectively (Figure 1A and 1B, not shown) confirming previous results.6,17 Lack of functional Syk also resulted in abolished platelet aggregation in response to GPVI or CLEC-2 agonists (Figure 1C and not shown). However, outside-in signaling of integrin αIIBβ3 as assessed in a platelet-spreading assay was unaffected in Syk-deficient platelets (Figure 2).

A combined GPVI and CLEC-2 deficiency dramatically affects hemostasis,18 whereas no such defect was observed in a small group of bone marrow chimeric Syk-deficient mice.19 In line with the latter report, Sykfl/fl, Pf4-cre+/− mice displayed moderately prolonged tail bleeding times (Figure 3A). However, occlusive thrombus formation on mechanical injury of the abdominal aorta was abrogated in Sykfl/fl, Pf4-cre+/− mice (Figure 3B), confirming an important role for Syk in arterial thrombosis. To assess the role of platelet Syk in brain infarction after focal cerebral ischemia, mice were subjected to 60-minute transient middle cerebral artery occlusion (mCAO), and infarct volume and neurological deficits were assessed after 24 hours. Strikingly, the infarct volumes in Sykfl/fl, Pf4-cre+/− mice were significantly reduced compared with wild-type mice (Figure 3C and 3D). Motor function, as assessed using the grip test, displayed a tendency toward improved outcome (Figure 3E), and the Bederson score assessing global neurological function was significantly improved (Figure 3F). Remarkably, no intracerebral hemorrhages were observed in these animals by systematic microscopical inspection (not shown). Thus, Syk deficiency in platelets provides protection against occlusive arterial thrombus formation and cerebral infarct progression, indicating that Syk might be an attractive antithrombotic/antithromboinflammatory target.

To test this directly, we used commercially available inhibitors, but the efficacy in vivo was limited. Therefore, we capitalized on the novel and highly selective oral ATP-competitive Syk inhibitor, B11002494.20 In vitro, 150 nmol/L B11002494 abrogated collagen-related peptide responses of wild-type platelets, whereas a concentration of 500 nmol/L was required to completely abolish convulxin and rhodocytin responses (Figure 4A and 4B). In contrast, no effects on G-protein–coupled receptor signaling were observed with any of the tested inhibitor concentrations (Figure 4A and 4B). B11002494-treated mouse platelets were able to spread normally on a fibrinogen-coated surface, but interestingly outside-in signaling of integrin αIIBβ3 in human platelets was impaired on inhibitor treatment (Figure 5 and not shown). Oral administration of a single dose of the inhibitor (100 mg/kg) 1 hour before blood drawing led to inhibition of platelet aggregation on stimulation of (hem)ITAM receptors (data not shown). However, these results were variable, and to maintain a constant plasma exposure for a longer time period, mice were treated twice, 15 hours and 1 hour before the experiments. To perform in vivo experiments, plasma exposure of the inhibitor was required for a longer time period. Oral administration of B11002494 (100 mg/kg BID) in mice resulted in plasma concentrations >980 nmol/L during a 24-hour time period (D. Lamb et al, unpublished data, 2015). Subsequent ex vivo analysis of platelet-rich plasma showed abolished GPVI-triggered platelet aggregation, whereas rhodocytin responses were reduced, but not abolished (Figure 4C). Interestingly, tail

| Table. Basal Blood Parameters of Sykfl/fl, Pf4-cre+/− (WT) and Sykfl/fl, Pf4-cre−/− (KO) Mice |
|-------------------------------------------------|---------------------------------|
| **Platelets, nL−1**                               | **Mean platelet volume, fL**    |
| Sykfl/fl, Pf4-cre+/− (WT)                         | 1097±69                         |
|                                                 | 5.4±0.1                         |
| Sykfl/fl, Pf4-cre−/− (KO)                        | 1134±136                        |
|                                                 | 5.4±0.1                         |
| **White blood cells, nL−1**                      | **Red blood cells, pl−1**       |
|                                                 | 6.0±2.1                         |
|                                                 | 9.1±0.7                         |
| **Hemoglobin, g/dL**                             | **Hematocrit, %**               |
|                                                 | 14.8±1.6                        |
|                                                 | 47.8±4.7                        |
| **Mean red blood cell volume, fL**               | **Mean red blood cell volume, fL** |
|                                                 | 54.2±1.6                        |
|                                                 | 54.6±1.0                        |

KO indicates knockout; and WT, wild-type.
bleeding times were unaltered in inhibitor-treated mice compared with vehicle-treated controls (Figure 6A).

B11002494-treated mice were subjected to mechanical injury of the aorta and found to be completely protected from vessel occlusion (Figure 6B). Next, wild-type mice were pretreated with B11002494 and then subjected to 60-minute tMCAO to determine the effect of the Syk inhibitor on ischemia–reperfusion injury. Similar to Sykfl/fl, Pf4-cre+/− mice, these animals displayed markedly reduced infarct sizes and improved motor function 24 hours after tMCAO but no evidence of major intracerebral bleeding (Figure 6C, not shown). To assess if the Syk inhibitor could be used therapeutically in an experimental setting, that is, when given after ischemia, mice were treated with the inhibitor directly after tMCAO (directly on removal of the filament), mimicking patients undergoing thrombectomy and subsequent treatment. Strikingly, also under these conditions, inhibitor-treated mice had reduced infarct sizes and a better motor function as compared with the vehicle-treated controls (Figure 6C–6F). Together, these results indicate that Syk inhibition might be effective in preventing cerebral reperfusion injury in patients after successful thrombectomy.

Figure 1. Abolished (hem)immunoreceptor tyrosine-based activation motif signaling in the absence of spleen tyrosine kinase (Syk). Flow cytometric analysis of (A) αIIbβ3 integrin activation (JON/A-PE) and (B) degranulation-dependent P-selectin exposure in response to the indicated agonists of Sykfl/fl, Pf4-cre−/− (wild-type [WT]; black bars) and Sykfl/fl, Pf4-cre+/− (knockout [KO]; gray bars) platelets. Results are mean fluorescence intensities (MFI)±SD of 5 mice per group. C, Platelet-rich plasma of WT (black line) and KO (gray line) mice was stimulated with the indicated agonists, and light transmission was recorded on a Born aggregometer. Representative aggregation curves of 3 individual experiments are shown. ***P<0.001. ADP indicates adenosine diphosphate; CRP, collagen-related peptide; Rhod, rhodocytin; and U46, U46619.

Figure 2. Washed platelets from wild-type (WT) and knockout (KO) mice were allowed to adhere to immobilized human fibrinogen (100 µg/mL). Images were taken at the indicated time points, and representative images are shown. Results indicate percentage of phase abundance with (1) round platelets, (2) only filopodia, and (3) filopodia and lamellipodia. Bar=5 µm.
Discussion

The unaltered bleeding times in BI1002494-treated mice are in line with observations made with other Syk inhibitors, such as fostamatinib or PRT060318, and strongly suggest that Syk inhibition might be a safe antithrombotic regimen. However, as with all preclinical studies, differences between

Figure 3. Spleen tyrosine kinase (Syk) deficiency protects from arterial thrombosis and ischemic stroke. A, Tail bleeding times of Syk<sup>KO</sup>, Pf4-Cre<sup>−/−</sup> and Syk<sup>KO</sup>, Pf4-Cre<sup>+/−</sup> mice. Each symbol represents 1 animal. B, In an aorta occlusion model, the blood flow was monitored for 30 min or until complete occlusion occurred. Time to stable vessel occlusion is depicted. Each symbol represents 1 animal. C, Mice were subjected to 60 min of transient middle cerebral artery occlusion (tMCAO). Representative images of 3 coronal sections stained with 2,3,5-triphenyltetrazolium chloride 24 h after tMCAO. White arrows highlight the infarcted area. D, Brain infarct volumes (n=9) of Syk<sup>KO</sup>, Pf4-Cre<sup>−/−</sup> (black bar) and Syk<sup>KO</sup>, Pf4-Cre<sup>+/−</sup> (gray bar) mice were measured by planimetry. Results represent means±SD. Each symbol represents 1 animal; not significant, *P>0.05; **P<0.05; ***P<0.01; ****P<0.001. KO indicates knockout; and WT, wild-type.

Figure 4. Pharmacological inhibition of spleen tyrosine kinase abolishes (hem)immunoreceptor tyrosine-based activation motif signaling. Dose–response curves showing flow cytometric analysis of (A) αIIbβ3 integrin activation (JON/A-PE) and (B) degranulation-dependent P-selectin exposure of wild-type platelets in response to the indicated agonists and the indicated concentrations of BI1002494. Results are percentage of inhibition±SD compared with vehicle-treated platelets. C, Platelet-rich plasma of vehicle-treated (black line) and BI1002494-treated (gray line) mice was stimulated with the indicated agonists, and light transmission was recorded on a Born aggregometer. Representative aggregation curves of 3 individual experiments are shown. ADP indicates adenosine diphosphate; CRP, collagen-related peptide; CVX, convulxin; Rhod, rhodocytin; and U46, U46619.
animal studies and the human situation need to be considered. One notable difference is the expression of FcγRIIA, which is present on human but not on mouse platelets. This difference in expression is the most likely explanation for the observed differences between human and mouse platelet spreading on inhibitor treatment because FcγRIIA supports platelet spreading in human platelets. Besides its effect on FcγRIIA, BI1002494 dose dependently abolished (hem)ITAM signaling in platelets, wherein collagen-related peptide–induced platelet activation seemed to be more sensitive toward inhibition than convulxin or rhodocytin-induced platelet activation. Different clustering capacities or variation in receptor-binding sites of the different agonists might contribute to these different sensitivities.21 Similar agonist and signaling pathway–dependent differences in BI1002494 efficacy were observed in various immune cells.20 Furthermore, GPVI and CLEC-2 signaling differ in the sequential order of Src family kinases and Syk,6 which could explain the different potencies of BI1002494 for inhibiting GPVI and CLEC-2 signaling and thereby the unaltered tail bleeding times in inhibitor-treated mice compared with the slightly prolonged bleeding times in Syk-deficient mice. However, given previous reports that the concomitant

Figure 5. Vehicle (veh)-treated and BI1002494 (Bl)-treated washed human platelets were allowed to adhere to immobilized human fibrinogen (100 µg/mL). Images were taken at the indicated time points, and representative images are shown. Results indicate percentage of phase abundancy with (1) round platelets, (2) only filopodia, (3) filopodia and lamellipodia, and (4) full platelet spreading. Bar=5 µm.

Figure 6. Pharmacological inhibition of spleen tyrosine kinase protects from occlusive thrombus formation and ischemic stroke. A, Tail bleeding times of vehicle-treated and BI1002494-treated mice. Each symbol represents 1 animal. B, In an aorta occlusion model, the blood flow was monitored for 30 min or until complete occlusion occurred. Time to stable vessel occlusion is depicted. Each symbol represents 1 animal. C, Mice were subjected to 60 min of transient middle cerebral artery occlusion (tMCAO). Brain infarct volumes (n=7–8) of vehicle-treated (black bars) and prophylactic or therapeutic BI1002494-treated (gray bars) mice were measured by planimetry. Results represent mean±SD. D, Representative images of 3 coronal sections of vehicle-treated mice and therapeutic BI1002494-treated mice stained with 2,3,5-triphenyltetrazoliumchloride 24 h after tMCAO. White arrows highlight the infarcted area. E, Grip test and (F) Bederson score of vehicle-treated mice and therapeutic BI1002494-treated mice determined 24 h after tMCAO. Each symbol represents 1 animal. not significant, P>0.05; *P<0.05; **P<0.01; ***P<0.001.
lack of GPVI and CLEC-2 severely compromises hemostasis, this could also point toward signaling-independent functions of the 2 (hem)ITAM receptors in hemostasis. Although our studies indicate that short-term inhibition of Syk is well tolerated, a longer treatment should be carefully considered because Syk deficiency could result in a blood–lymphatic mixing phenotype or defective integrity of high endothelial venules, indicating that chronic Syk inhibition might have a similar effect in mice and possibly humans. However, blood–lymphatic mixing or blood-filled lymph nodes were not observed in the Syk-deficient mice used for our studies. It was of our interest to determine the role of Syk in ischemia–reperfusion injury, a phenomenon that often occurs on recanalization of occluded cerebral arteries in the setting of acute stroke. The tMCAO model is a well-established model to study this process and clinically reflects thrombectomy. The therapeutic setting, in which mice were treated with BI1002494 directly on removal of the filament, would reflect a clinical situation where a patient undergoes successful thrombectomy with additional treatment with an antplatelet agent. In the Syk-deficient mice, the neurological outcome was significantly improved, whereas only a tendency toward a better motor function was observed. In contrast, the inhibitor-treated mice have a significantly improved motor function. However, a clear reduction of infarct sizes was observed for both Syk-deficient and inhibitor-treated mice, and this was reflected by the improved outcomes in the neurological and motor function tests. In addition, our data were obtained using mouse models, and when translating the results to a clinical situation, other risk factors, besides dysregulated platelet function, such as atherosclerosis, diabetes mellitus, hypertension, and obesity have to be taken into account. In conclusion, we could demonstrate that pharmacological inhibition of Syk results in efficient and safe protection from arterial thrombosis and acute ischemic stroke without affecting hemostasis.

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References


Highlights

- The spleen tyrosine kinase is an essential signaling mediator downstream of the platelet collagen receptor glycoprotein VI and C-type lectin-like receptor 2.
- Spleen tyrosine kinase in platelets is required for infarct progression in ischemic stroke.
- Spleen tyrosine kinase deficiency does not affect hemostasis.
- Pharmacological inhibition of spleen tyrosine kinase protects from occlusive thrombus formation and ischemic stroke.
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The novel oral Syk inhibitor, Bl1002494, protects mice from arterial thrombosis and thrombo-inflammatory brain infarction

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MATERIAL AND METHODS

Mice
C57BL/6JRj mice were obtained at an age of 6 to 9 weeks (Janvier Labs, Saint-Berthevin, France). Sykfl/fl mice, which have been backcrossed 12 times with C57BL/6JRj mice, were intercrossed with mice carrying the Cre-recombinase under the Pf4 promoter to generate megakaryocyte and platelet-specific Syk knock-out mice (Sykfl/fl, Pf4-cre/).1 Cre-negative (Sykfl/fl, Pf4-cre/-) littermates served as controls. All mice used in experiments were 8 to 12 weeks old and sex-matched. Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken). The number of animals used is indicated in the figure legends.

Compound preparation
For in vitro assays, BI1002494 (C23H24N3O5),2 kindly provided by Boehringer Ingelheim, was dissolved in dimethyl sulfoxide (DMSO) and final DMSO concentration in the assays was less than 0.1%. The washed platelets were incubated at 37°C with BI1002494 10 minutes prior to the experiment. For animal experiments, BI1002494 was dissolved in water and applied by oral gavage 15 hours and 1 hour prior to blood taking (ex vivo aggregation experiments) or the in vivo experiments (100 mg/kg b.i.d.). To assess the therapeutic function of the Syk inhibitor, BI1002494 was dissolved in water and applied by oral gavage directly after tMCAO (100 mg/kg), i.e. directly upon removal of the filament.

Reagents and antibodies
BI1002494 was kindly provided by Boehringer Ingelheim.2 ADP, apyrase, fibrinogen, indomethacin (Sigma-Aldrich, Schnelldorf, Germany), thrombin (Roche Diagnostics, Mannheim, Germany), convulxin and U46619 (both Enzo Life Sciences, Lörrach, Germany) were purchased. Collagen-related peptide (CRP) was generated as previously described.3 Rhodocytin was purified from Calloselasma rhodostoma venom and was kindly provided by J. Eble. The anesthetic drugs medetomidine (Pfizer, Karlsruhe, Germany), midazolam (Roche Diagnostics, Mannheim, Germany), fentanyl (Janssen-Cilag, Neuss, Germany) and the antagonists atipamezole (Pfizer, Karlsruhe, Germany), flumazenil and naloxone (AlleMan Pharma, Pfullingen, Germany) were used according to the regulations of the local authorities. JON/A-PE, specifically recognizing the high-affinity conformation of the integrin αIIbβ3 was from Emfret Analytics (Eibelstadt, Germany). All other antibodies were generated and modified in our laboratory.
Platelet preparation

Mice were bled under isoflurane anesthesia from the retro-orbital plexus into a tube containing 20 U/ml heparin (Ratiopharm, Ulm, Germany). Platelet-rich plasma (PRP) was obtained by two centrifugation steps at 300 g for 7 minutes at room temperature (RT). For the preparation of washed platelets, PRP was centrifuged at 700 g for 5 minutes at RT and the pellet was resuspended and washed in calcium-free Tyrode’s buffer containing 0.1 µg/ml prostacyclin (PGI₂, Sigma-Aldrich, Schnelldorf, Germany) and apyrase (0.02 U/ml, Sigma-Aldrich, Schnelldorf, Germany). For human platelets, fresh blood samples of healthy volunteers were collected in 1/10 volume of acid-citrate-dextrose and centrifuged at 200 g for 10 minutes at RT. PRP was collected and centrifuged at 800 g for 10 minutes at RT. The pellet was resuspended and washed in calcium-free Tyrode’s buffer containing 0.1 µg/ml PGI₂ and apyrase (0.02 U/ml). The platelet count was determined using a Sysmex KX-21N automated hematology analyzer (Sysmex Corporation, Kobe, Japan). To adjust the platelet concentration after centrifugation, the pellet was resuspended in calcium-free Tyrode’s buffer containing apyrase (0.02 U/ml) and the platelet suspension was allowed to rest for at least 30 minutes at 37°C prior to the experiment.

Determination of blood parameters

To determine blood parameters, 50 µl blood were drawn from the retro-orbital plexus of anesthetized mice using heparinized capillaries and collected into a tube containing 300 µl heparin in TBS (20 U/ml, pH 7.3). Blood cell counts and parameters were determined using a Sysmex KX-21N automated hematology analyzer (Sysmex Corporation, Kobe, Japan).

Determination of platelet surface protein expression and platelet activation by flow cytometry

To determine platelet surface protein expression, 50 µL blood were collected from the retro-orbital plexus in heparin-containing tubes, diluted 1:20 with calcium-free Tyrode’s buffer and stained for 15 minutes at RT with saturating amounts of fluorophore-conjugated antibodies and immediately analyzed on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). For platelet activation, samples were activated with agonists at the indicated concentrations, stained with fluorophore-conjugated antibodies at saturating concentrations for 15 minutes at 37°C and directly analyzed on a FACSCalibur.

Aggregometry

PRP was diluted into Tyrode’s buffer containing 2 mM calcium. Agonists were added at the indicated concentrations to the continuously stirring (1000 rpm) platelet suspension. Light transmission was measured using a Fibrintimer 4-channel aggregometer (APACT...
Laborgeräte und Analysensysteme) for 10 minutes and was expressed in arbitrary units with buffer representing 100% transmission and PRP representing 0% transmission.

**Platelet adhesion on fibrinogen under static conditions**

Glass coverslips (24 x 60 mm) were coated with 100 µg/ml human fibrinogen diluted in sterile 1x PBS o/n at 4°C under humid conditions. Coverslips were blocked with sterile filtered 1% BSA in PBS for 2 h at RT and rinsed with Tyrode’s buffer containing 2 mM Ca²⁺. Syk deficient platelets were treated with indomethacin (1.4 µM) and apyrase (1 U/ml) to inhibit the effect of the second wave mediators thromboxane A2 and ADP. Then, human platelets and mouse platelets were allowed to spread for the indicated time points at RT. Platelets were fixed with 4% PFA in PBS for 5 min at RT and visualized with a Zeiss Axiovert 200 inverted microscope (100x oil objective, Zeiss, Oberkochen, Germany) using differential interference contrast (DIC) microscopy. Representative images were taken and evaluated according to different platelet spreading stages using ImageJ software (NIH, Bethesda, MD, USA).

**Tail Bleeding Time**

Mice were anesthetized by intraperitoneal injection of medetomidine (0.5 µg/g), midazolam (5 µg/g) and fentanyl (0.05 µg/g) and a 1-mm segment of the tail tip was removed using a scalpel. Tail bleeding was monitored by gently absorbing blood on filter paper at 20 second intervals, without interfering with the wound site. Bleeding was determined to have ceased when no blood was observed on the paper. Experiments were stopped manually after 20 min by cauterization to prevent excessive blood loss and anesthesia was antagonized by intraperitoneal injection of atipamezole (2.5 µg/g), flumazenil (0.05 µg/g) and naloxone (1.2 µg/g).

**Aorta occlusion model**

Mice were anesthetized by intraperitoneal injection of medetomidine (0.5 µg/g), midazolam (5 µg/g) and fentanyl (0.05 µg/g) and a longitudinal incision was used to open the abdominal cavity and expose the abdominal aorta of anesthetized mice. An ultrasonic flow probe (0.5PSB699, Transonic Systems, New York, USA) was placed around the vessel and thrombus formation was induced by a single firm compression with a forceps upstream of the flow probe. Blood flow was monitored until complete blood vessel occlusion occurred for at least 5 min or for a maximum of 30 min.
**Transient middle cerebral artery occlusion (tMCAO)**

Focal cerebral ischemia was induced in 8-to-12-week-old mice by a transient middle cerebral artery occlusion (tMCAO) as previously described. Inhalation anesthesia was induced by 2% isoflurane in a 70% N₂/30% O₂ mixture and a servo-controlled heating device was used to record and maintain body temperature during the surgical procedure. The duration of the surgical procedure per animals was kept below 15 minutes. A silicon rubber-coated 6.0 nylon monofilament (6021PK10, Doccol, Redlands, CA, USA) was advanced through the carotid artery up to the origin of the middle cerebral artery (MCA) causing an MCA infarction. After an occlusion time of 60 min, the filament was removed allowing reperfusion. Animals were sacrificed 24 h after reperfusion and brains were checked for intracerebral hemorrhages. The extent of infarction was quantitatively assessed 24 hours after reperfusion on 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) (2% (w/v) solution) stained brain sections. Global neurological outcome and motor function were evaluated by the Bederson score and the grip test, respectively. Planimetric measurements of infarcted areas (ImageJ software, NIH, Bethesda, MD, USA) corrected for brain edema and assessment of functional outcome were performed in a blinded fashion.

**Data analysis**

Results from at least 3 experiments per group are presented as mean ± SD. Differences between two groups were assessed using the Student’s t-test. The Fisher’s exact test was applied to assess variance in occurrence of occlusion. P-values <0.05 were considered statistically significant with: ns, p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001.
REFERENCES


