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Selective inhibition of Panx1 channels decreases hemostasis and thrombosis *in vivo*

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ABSTRACT

Background: Hemostasis is a tightly regulated physiological process to rapidly induce hemostatic plugs at sites of vascular injury. Inappropriate activation of this process may lead to thrombosis, *i.e.* pathological blood clot formation in uninjured vessels or on atherosclerotic lesions. ATP release through Pannexin1 (Panx1) membrane channels contributes to collagen-induced platelet aggregation *in vitro*.

Objective: To investigate the effects of genetic and pharmacological inhibition of Panx1 on hemostasis and thrombosis *in vivo*.

Results: Bleeding time after tail clipping was increased by 2.5-fold in $Panx1^{-/-}$ mice compared to wild-type controls, suggesting that Panx1 deficiency impairs primary hemostasis. Wire myography on mesenteric arteries revealed diminished vasoconstriction in response to phenylephrine or U446619 in $Panx1^{-/-}$ mice. Mice with platelet-specific deletion of Panx1 ($Panx1^{PDel}$) displayed 2-fold longer tail bleeding times than $Panx1^{fl/fl}$ controls. Moreover, venous thromboembolism (VTE) after injection of collagen/epinephrine in the jugular vein was reduced in $Panx1^{-/-}$ and $Panx1^{PDel}$ mice. $Panx1^{PDel}$ mice also showed reduced $FeCl_3$-induced thrombosis in mesenteric arteries. BrilliantBlue-FCF, a Panx1 channel inhibitor, decreased collagen-induced platelet aggregation *in vitro*, increased tail bleeding time and reduced VTE in wild-type mice. Furthermore, we developed a specific Panx1 blocking antibody targeting a Panx1 extracellular loop, which reduced ATP release from platelets *in vitro*. Treating wild-type mice with this antibody increased tail bleeding time and decreased VTE compared to control antibody.

Conclusions: Panx1 channel deletion or inhibition diminishes clot formation during hemostasis and thrombosis *in vivo*. Blocking Panx1 channels may be an attractive strategy for modulating platelet aggregation in thrombotic disease.

1. Introduction

Pannexins (Panxs) form a three-membered family of glycoproteins, which establish membrane channels at the cell surface. Panx1 is expressed in most mammalian cells and tissues, including skeletal and smooth muscle, endothelium, leukocytes and adipocytes [1,2]. The general topology of Panxs features four transmembrane domains, two extracellular loops (ELs) and an amino-terminus, carboxy-terminus (CT) and intracellular loop all located in the cytoplasm. The CTs differ considerably in both length and composition and are unique to the Panx subtype [1,3]. Panx1 is synthesized, N-glycosylated and oligomerized into hexameric channels in the endoplasmic reticulum prior to further editing and delivery to the Golgi apparatus. Then, Panx1 channels traffic to the plasma membrane where they act as nucleotide release channels but likely serve additional roles in release or uptake of small molecules [3]. Released nucleotides, such as ATP, can signal by targeting surface receptors in a paracrine, autocrine, or even in an endocrine fashion, thus contributing to intercellular signaling and tissue homeostasis. Multiple factors induce Panx1 channel activation, including mechanical stretch, high extracellular K^+, metabotropic

Abbreviations: BB-FCF, BrilliantBlue-FCF; BSA, bovine serum albumine; CT, carboxy-terminus; ECs, endothelial cells; EL, extracellular loop; Panx, Pannexin; SFK, Src family kinase; VTE, venous thromboembolism; WT, wild-type

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receptor activation with associated elevated intracellular Ca^{2+} (*f.e.* P2Y receptors), and ionotropic-, chemokine-, and glycoprotein receptor activation resulting in Src family kinase (SFK)-mediated phosphorylation (*f.e.* GPVI receptors) [2].

P2 receptors play a critical role in platelet function. These receptors are subdivided into two groups that are represented by receptors sensitive to ATP (P2X1) and receptors sensitive to ADP (P2Y1 and P2Y12). The ATP-gated P2X1 receptors allow Ca^{2+} influx into the platelets, resulting in a transient shape change and platelet activation [4,5]. These receptors have been shown to induce and sustain ATP-mediated aggregation in response to collagen after vascular injury [6]. In addition, P2X1 is sensitive to low collagen concentrations in high shear stress conditions, which makes ATP an agonist of platelet aggregation at the early stage of arterial endothelium damage [7]. A role for Panx1 in fine-tuning collagen-induced platelet reactivity was recently shown [8,9]. Specifically, collagen binding to GPVI receptors drives a Src-dependent phosphorylation of Panx1 channels leading to the release of ATP and subsequent activation of P2X1 receptors resulting in human platelet aggregation. Furthermore, a single nucleotide polymorphism of Panx1 (Panx1-400A > C), inducing the expression of a gain-of-function Panx1 channel, was associated with increased collagen-induced aggregation in healthy volunteers [8]. Altogether, these observations suggest that Panx1 sets platelet reactivity. However, direct *in vivo* involvement of Panx1 in hemostasis and thrombosis remains to be shown.

2. Materials and methods

2.1. Animals

All animal studies were approved by the Swiss Federal Veterinary Office. *In vivo* tail bleeding, venous thromboembolism (VTE) and $FeCl_3$-induced mesenteric artery thrombosis experiments were performed as previously described [10–12] on age-matched male wild-type (WT), *Panx1*$^{-/-}$ [13], *Pf4Cre*^{Tg}*Panx1*$^{fl/fl}$ (*Panx1*^{PDel}) and *Panx1*$^{fl/fl}$ mice [14] on a C57BL6/J background. In some experiments, 100 μg/kg Brilliant-Blue-FCF (BB-FCF; Sigma-Aldrich) [15], vehicle, 1 μg/kg antibody HRB454 or HRB460 were retroorbitally administered in WT mice 7 min prior to the experiment. Whole blood cell counts were performed using a hematocytometer (Sysmex Digitana). Wire myography was performed on isolated mesenteric arteries using previously established protocols [16,17].

2.2. Immunofluorescent staining, quantitative PCR and platelet aggregation

Murine washed platelets were obtained as described previously [10]. In some experiments, preincubation with 1 mM BB-FCF or 100 μM PP2 (Tocris) was performed for 7 min before activation with 1 μg/mL collagen-I (Nycomed). Then, platelets or RAW264.7 macrophages (ATCC) were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton X-100 for 15 min blocked with 2% bovine serum albumin (BSA) and incubated overnight with an anti-Panx1$_{414–425}$ antibody [8] (1:500) or anti-Panx1$_{PhosphoY308}$ antibody (1:500; Millipore) followed by a 2-hour incubation with their respective secondary antibodies, counterstaining with Evans Blue, mounting (Vectashield; VectorLabs) and confocal imaging (Zeiss LSM800). Total RNA was extracted from platelets or lymphocytes from spleen and subjected to quantitative PCR using previously described protocols [14]. Aggregation responses of murine platelets were measured by turbidimetry (TA-8V; SD-Medical) as described elsewhere [8]. As tested previously [18], the blue color of the dye does not affect the outcomes of turbimetry.

2.3. Human washed platelets and ATP release measurement

Blood samples were collected from healthy volunteers at the blood bank donor facility of the Geneva University Hospitals and extraction of washed platelets followed a previously described protocol [8]. Platelets were preincubated during 7 min with 1 μg/mL HRB454 or HRB460 antibodies. Then, platelets were activated during 6 min with 1 μg/mL collagen-I. Supernatants were collected after centrifugation and ATP concentrations were determined with a bioluminescent assay kit (Sigma-Aldrich).

2.4. Production and validation of Panx1 blocking antibodies

HRB454 and HRB460 antibodies [19] targeting murine Panx1-EL1 (aa 74-83) and Panx1-CT (aa 414-425) were produced by the Geneva Antibody Facility (https://www.unige.ch/medecine/antibodies/) as mini-antibodies with the antigen-binding scFv fused to a human Fc according to established protocols [20]. Antibodies were validated by ELISA to bind the correct peptidic sequences, and by FACS and immunostaining to recognize Panx1 in RAW264.7 macrophages. Thus, cells were fixed with 4% paraformaldehyde for 15 min, blocked with 2% BSA or Fc-block, incubated overnight with HRB454 (1:250) or HRB460 (1:250) followed by 1–2-hour incubation with secondary antibody. Of note, the targeting sequence of HRB454 is completely conserved between mouse and human Panx1.

2.5. Statistical analyses

Data were analyzed using GraphPad Prism6 software and shown as mean ± SEM. Comparisons were performed using *t*-test, Mann-Whitney *U* test or ANOVA followed by Bonferroni's post-tests, where appropriate. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Ubiquitous Panx1 deletion decreases hemostasis in mice

Panx1 immunosignal was detected in platelets from WT mice and was absent in *Panx1*$^{-/-}$ platelets (Fig. 1A). After vascular injury, local endothelium-derived factors induce a transient vasoconstriction followed by platelet adherence to the exposed extracellular matrix, which induces platelet activation, release of their secretory granules and recruitment of additional platelets to form a hemostatic plug. To study the role of Panx1 in primary hemostasis *in vivo*, we measured the time to bleeding cessation after transverse amputation of the tail tip. Bleeding time was 2.5-fold higher in *Panx1*$^{-/-}$ mice to that of WT mice (Fig. 1B,C).

As endothelial Panx1 has been shown to modulate the severity of ischemic stroke by controlling cerebral inflammation and myogenic tone [21], we compared vasomotor responses in mesenteric arteries of WT and *Panx1*$^{-/-}$ mice. We found impaired vasocontraction in response to phenylephrine or to the thromboxane A_2 analogue U446619 in *Panx1*$^{-/-}$ mice (Fig. 1D,E). These results support earlier studies showing that Panx1 facilitates the contraction of murine resistance arteries by associating with the α1-adrenergic receptors in smooth muscle cells, whereas vasoconstriction in response to serotonin or endothelin-1 is independent of Panx1 [22,23]. Endothelium-dependent relaxation in response to acetylcholine as well as endothelium-independent relaxation in response to sodium nitroprusside were unaffected in *Panx1*$^{-/-}$ mice (Fig. 1F,G).

In addition to platelets and endothelial cells (ECs), erythrocytes and leukocytes may participate in the hemostatic process. Therefore, we quantified the number of these cells in peripheral blood from WT and *Panx1*$^{-/-}$ mice. Hematocrit, number of lymphocytes and platelets were not different between the genotypes (Fig. 1H–J).

Altogether, these results show that *Panx1*$^{-/-}$ mice have increased bleeding time *in vivo*, however whether the effect is attributable to differences in initial vasoconstriction or in platelet function remains to be investigated.

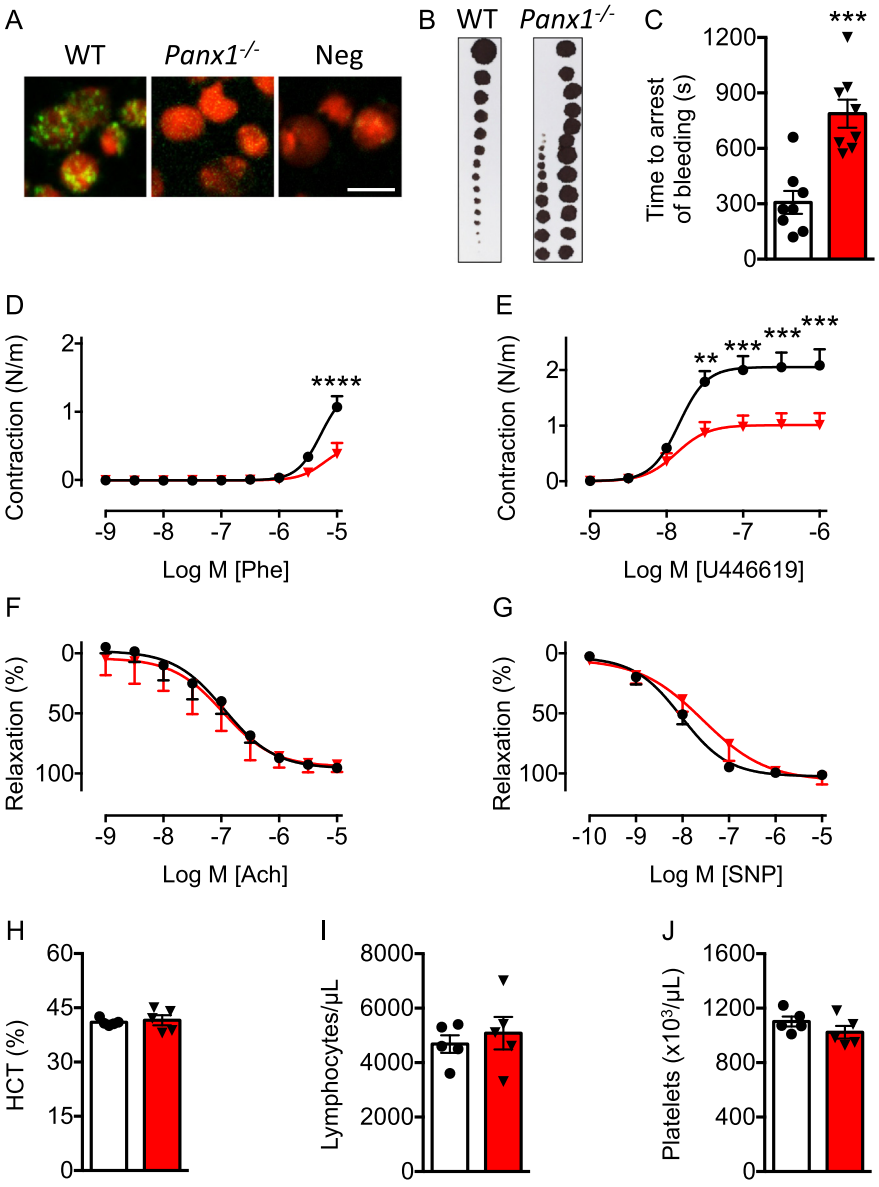

Fig. 1. Ubiquitous Panx1 deletion delays hemostasis. **A**. Immunofluorescent staining for Panx1 (in green) on platelets from WT or Panx1$^{-/-}$ mice. Platelets were counterstained with Evans Blue (in red). Negative control (Neg) included omission of first antibody. Scale bar represents 5 µm. **B**. After 2 mm tail-tip amputation, the time to bleeding cessation was determined by applying a Whatman filter paper to the blood drop every 30 s until bleeding stopped. **C**. Quantification of time to bleeding cessation in WT (white) or Panx1$^{-/-}$ (red) mice ($N = 8$). **D–G**. Wire myography to measure contraction and/or relaxation of mesenteric arteries of WT (black) or Panx1$^{-/-}$ (red) mice ($N = 6$) in response to phenylephrine (**D**) or U446619 (**E**). Relaxation induced by acetylcholine (**F**) or sodium nitroprusside (**G**) was measured in phenylephrine-precontracted mesenteric arteries of WT or Panx1$^{-/-}$ mice ($N = 6$). Hematocrit (**H**), number of lymphocytes (**I**) or platelets (**J**) were determined in whole blood of WT (white) or Panx1$^{-/-}$ (red) mice ($N = 5$). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Specific deletion of Panx1 from platelets delays hemostasis in vivo

Pf4-Cre transgenic mice allow for the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function *in vivo* [24]. We generated *Pf4Cre*^{Tg}*Panx1*$^{fl/fl}$ mice (hereafter called *Panx1*^{PDel}) and confirmed the efficiency and specificity of the deletion by quantitative PCR on platelets and lymphocytes. While *Panx1*$^{fl/fl}$ platelets did not show any difference in Panx1 mRNA levels compared to that of WT platelets, Panx1 was absent from *Panx1*^{PDel} and *Panx1*$^{-/-}$ platelets (Fig. 2A). Furthermore, comparable Panx1 mRNA levels were found in lymphocytes of *Panx1*^{PDel}, *Panx1*$^{fl/fl}$ and WT mice while Panx1 expression was absent in lymphocytes of *Panx1*$^{-/-}$ mice (Fig. 2B). Immunofluorescent staining confirmed absence of Panx1 protein from *Panx1*^{PDel} platelets and its presence in platelets of *Panx1*$^{fl/fl}$ control mice (Fig. 2C). Platelet-specific Panx1 deletion increased the time to cessation of tail bleeding by 2-fold (Fig. 2D), suggesting that the increased bleeding time in *Panx1*$^{-/-}$ mice is largely due to impaired platelet function, and that vasoconstriction plays only a minor role in the hemostatic response of *Panx1*$^{-/-}$ mice. Nevertheless, further studies are warranted to evaluate the possible effects of Panx1 deficiency on thrombosis *in vivo*.

3.3. Platelet-specific Panx1 deletion decreases thrombosis in mice

We have recently demonstrated that collagen binding to GPVI receptors induces Src-dependent phosphorylation and activation of Panx1 channels *in vitro*. This in turn leads to a Panx1-dependent ATP release and subsequent activation of P2X1 receptors promoting human platelet aggregation [8]. To study the effect of Panx1 deletion in a context where collagen-induced platelet aggregation is independent from ECs, we induced VTE by injecting a mix of collagen/epinephrine in the jugular vein of anesthetized mice and measured the time to respiratory arrest. *Panx1*$^{-/-}$ and *Panx1*^{PDel} mice displayed an increased time to respiratory arrest as compared to their respective controls (Fig. 2E,F), suggesting delayed venous thrombosis and pulmonary embolism upon Panx1 deficiency. Next, we investigated the effects of platelet-specific Panx1 deletion in mice on clot formation in $FeCl_3$-injured mesenteric arteries using intravital microscopy. Interestingly, both the time to initial thrombus formation (Fig. 2G) and the time to arterial occlusion (Fig. 2H) tend to increase in *Panx1*^{PDel} mice compared to *Panx1*$^{fl/fl}$ controls. Altogether, our data support the idea that Panx1 might be a potential *in vivo* target to modulate platelet aggregation induced by collagen.

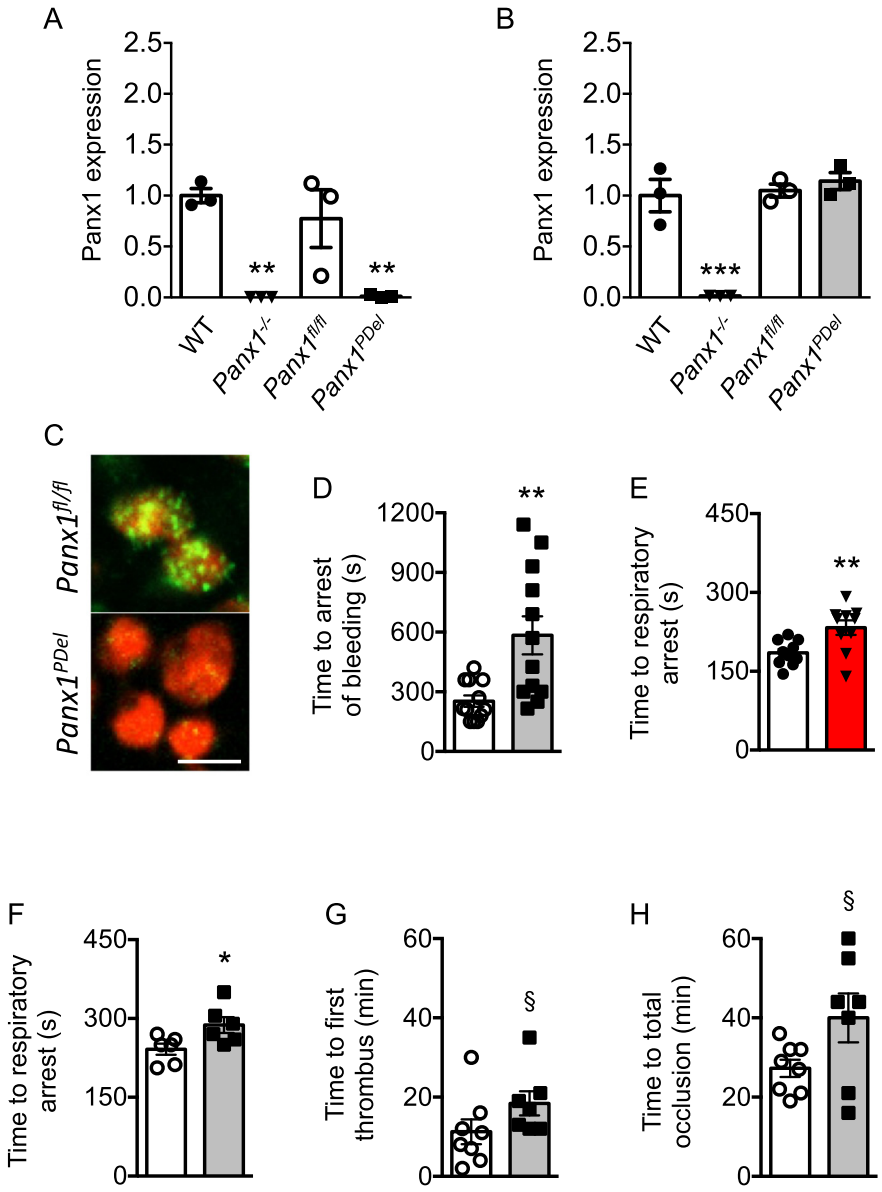

Fig. 2. Platelet-specific deletion of Panx1 delays hemostasis and thrombosis.

A–B. Panx1 mRNA expression in platelets (**A**) and lymphocytes (**B**) of WT, Panx1$^{-/-}$, Panx1$^{fl/fl}$ and Panx1^{PDel} mice ($N = 3$) was assessed by quantitative PCR. **C**. Immunofluorescent staining for Panx1 (in green) on platelets from Panx1$^{fl/fl}$ and Panx1^{PDel} mice. Platelets were counterstained with Evans Blue (in red). Scale bar represents 5 μm. **D**. Time to cessation of tail bleeding was quantified in Panx1$^{fl/fl}$ (white) or Panx1^{PDel} (grey) mice ($N = 12$). **E–F**. VTE was induced by injecting a mixture of collagen/epinephrine into the jugular vein. Quantification of time to respiratory arrest in (**E**) WT (white) or Panx1$^{-/-}$ (red) mice ($N = 10$) and (**F**) Panx1$^{fl/fl}$ (white) or Panx1^{PDel} (grey) mice ($N = 6$). **G–H**. Intravital microscopy was used to assess the time to initial thrombus formation (**G**) and the time to total occlusion (**H**) in $FeCl_3$-injured mesenteric arteries of Panx1^{PDel} (grey) or Panx1$^{fl/fl}$ (white) mice ($N = 7$–8). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{§}P = 0.06$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Blocking Panx1 channels delays hemostasis and thrombosis in mice

The lack of chemical compounds that selectively act on Panx1 channel gating hampers Panx1 research. Currently used pharmacological Panx1 blockers, such as carbenoxolone, mefloquine and probenecid, often also block connexin channels or act on purinergic receptors [25,26]. The best option seems to be ^{10}Panx1, an EL1 mimetic peptide that impedes the passage of small molecules and ATP through the Panx1 channel [27]. However, such peptides display poor serum stability limiting *in vivo* applicability.

BB-FCF is a relatively new Panx1 channel blocking compound that inhibits Panx1 channels as shown by patch clamp and ATP release measurements [28]. Moreover, it has been used for the marking of saphenous vein grafts, in which it abrogates response to vascular injury [29]. Furthermore, Panx1 channel inhibition with BB-FCF reduces collagen-induced aggregation of human platelets without toxic side-effects [18]. Thus, we assessed the function of murine platelets by turbidimetry after 7 min preincubation with BB-FCF. A high dose of BB-FCF (1 mM) completely inhibited collagen-induced aggregation (Fig. 3A,B), whereas a lower dose (100 μM) was without effect, pointing to a narrow range of effectiveness of the compound. As inhibition of collagen-induced aggregation of human platelets involved phosphorylation of Panx1 by SFKs, we performed immunostaining on murine platelets using an antibody recognizing the Src kinase phosphorylation site on Panx1 (Panx1^{Y308}) [30]. Panx1^{Y308} phosphorylation was increased after platelet activation with collagen as compared to non-activated platelets (Fig. 3C, upper left panels), illustrating that Panx1-induced inhibition of collagen-induced platelet aggregation involves a similar mechanism in human and mouse platelets, as expected. Although lysates of resting WT and Panx1-deficient platelets contained similar ATP concentrations (95.5 ± 12.3 nM *vs.* 106.6 ± 5.9 nM, respectively, $N = 9$), lysates of activated Panx1-deficient platelets contained 38% more ATP than lysates of activated WT platelets (65.9 ± 3.7 nM *vs.* 47.8 ± 5.2 nM, respectively; $P = 0.01$). This reduction in ATP release by Panx1-deficient platelets further underlines that mouse and human platelets use similar activation pathways involving Panx1 upon activation by collagen.

Preincubation of platelets with the Src-kinase inhibitor PP2 abolished Panx1 phosphorylation. Interestingly, preincubation with 1 mM BB-FCF did not inhibit collagen-induced Panx1^{Y308} phosphorylation, suggesting that BB-FCF acts extracellularly on Panx1 channel permeation rather than by affecting phosphorylation (Fig. 3C, upper right

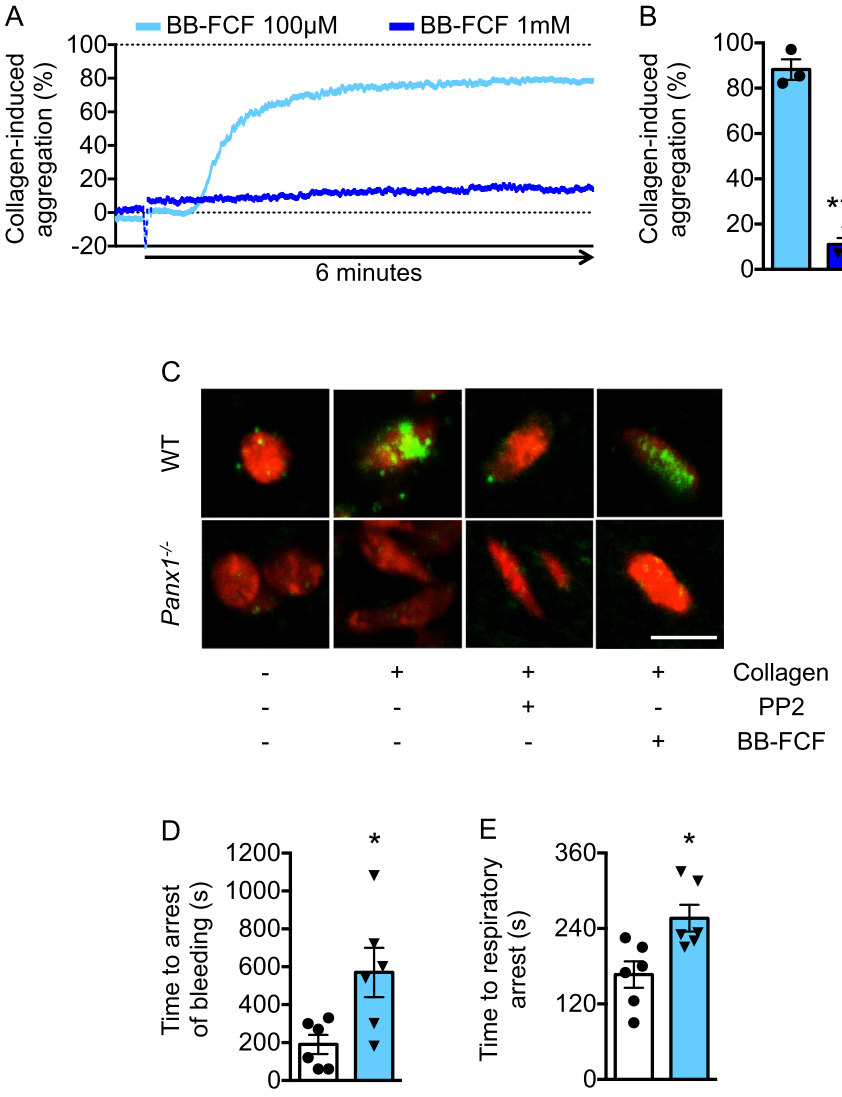

Fig. 3. Blocking Panx1 channels delays hemostasis and thrombosis in mice.
A–B. Representative traces and quantification of collagen-induced aggregation of WT mouse platelets pre-incubated for 7 min with the Panx1 blocker BB-FCF at 1 mM (dark blue; $N = 3$) or 100 μM (light blue; N = 3). **C.** Phosphorylation of Panx1^{Y308} was detected by immunofluorescent staining (in green) on platelets from WT or Panx1$^{-/-}$ mice activated during 1 min with 1 μg/mL collagen after 7 min preincubation with 100 μM PP2 or 1 mM BB-FCF. Platelets were counterstained with Evans Blue (in red). Scale bar represents 5 μm. **D.** Quantification of time to cessation of tail bleeding in WT mice treated for 7 min with 100 μg/kg BB-FCF (light blue; $N = 6$) or vehicle (white; $N = 6$). **E.** Quantification of time to respiratory arrest in WT mice treated for 7 min with 100 μg/kg BB-FCF (light blue; $N = 6$) or vehicle (white; $N = 6$) prior to VTE induction. $^{*}P < 0.05$, $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

panel). The specificity of the anti-Panx1$_{PhosphoY308}$ antibody was assured by absence of staining in platelets from *Panx1*$^{-/-}$ mice (Fig. 3C, lower panels). Next, we performed intravenous injection of BB-FCF in WT mice and tested tail bleeding and VTE 7 min later. Bleeding time and time to respiratory arrest were increased after treatment with BB-FCF (Fig. 3D,E), demonstrating the feasibility of altering the hemostatic balance *in vivo* by compounds acting on Panx1 channels. Importantly, BB-FCF is rapidly excreted from the body and does not seem to display (serious) side-effects either [25]. However, similar to other pharmacological Panx1 inhibitors, the Panx1 specificity of BB-FCF has recently been called into question by studies showing that high concentrations of the dye can directly inhibit P2X receptors [31], and it can thus not be excluded that the *in vivo* effects of BB-FCF on hemostasis may be partly driven by inhibition of P2X7 receptors expressed in the endothelium. This further emphasizes the need for development of specific Panx1 channel blockers.

3.5. A Panx1-blocking antibody regulates hemostasis and thrombosis in vivo

In view of possible non-specific effects of the available Panx1 inhibitors, we generated a Panx1 blocking antibody recognizing the WRQAAFVDSY sequence in Panx1-EL1 (HRB454, hereafter called α-Panx1EL) together with a control antibody against part of the intracellular Panx1-CT (HRB460, hereafter called α-Panx1CT). As shown by ELISA, each antibody bound in a concentration-dependent manner only to the Panx1 peptide against which they were raised (Fig. 4A). Flow cytometry on non-permeabilized RAW264.7 macrophages revealed a strong immunofluorescence with α-Panx1EL but not with α-Panx1CT (Fig. 4B). Similarly, α-Panx1EL but not α-Panx1CT revealed Panx1 staining by fluorescence microscopy on non-permeabilized RAW264.7 cells (Fig. 4C). Furthermore, α-Panx1EL recognized Panx1 in platelets of WT mice, whereas *Panx1*$^{-/-}$ platelets were without signal, thus attesting antibody specificity (Fig. 4D). α-Panx1EL reduced collagen-induced ATP release from human platelets by half compared to α-Panx1CT (Fig. 4E), illustrating the specific blocking properties of α-Panx1EL. Interestingly, tail bleeding time and VTE were delayed in mice injected with α-Panx1EL compared to those of mice injected with α-Panx1CT (Fig. 4F,G).

Currently, antiplatelet drugs are the cornerstone in the prevention of atherothrombosis but their clinical benefit is limited by an increased risk for bleeding. *P2X1*$^{-/-}$ mice exhibit inhibition of thrombosis with no prolongation of the tail bleeding time [7], indicating a dispensable role of P2X1 function in hemostasis, and the collagen receptor GPVI and P2X1 have therefore been proposed as potential targets for a safe new class of anti-platelet drugs [32,33]. By their role upstream of P2X1 and downstream of GPVI in this signaling cascade, inhibition of Panx1 channels may be an attractive target to further fine-tune the regulation of this signaling pathway. Altogether, our data demonstrate that targeting Panx1 channels diminishes clot formation *in vivo* in collagen-dependent mouse models of arterial and venous thrombosis. It should

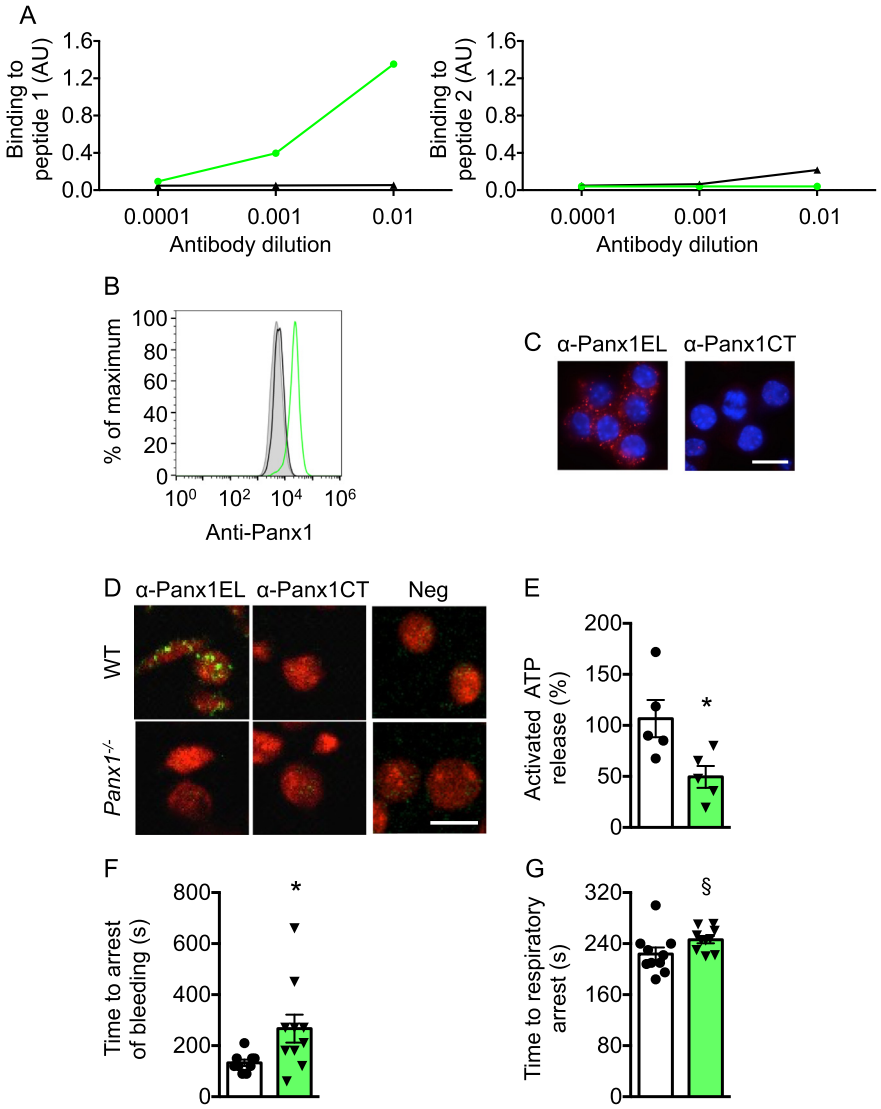

Fig. 4. Panx1-blocking antibody delays hemostasis and VTE in mice.

A. ELISA results demonstrating that HRB454 and HRB460 (α-Panx1CT, black) bind in a concentration-dependent manner only to the Panx1 peptide against which they were raised (peptide1 = WRQAAFVDSY; peptide2 = EKNSRQRLLNPS). **B**. Flow cytometry on RAW264.7 macrophages with α-Panx1EL or α-Panx1CT (black) antibodies. Control curve (secondary antibody only) in grey. **C**. Immunofluorescent staining for Panx1 (in red) on RAW264.7 macrophages. Nuclei were stained with DAPI (in blue). Scale bar represents 20 μm. **D**. Immunofluorescent staining for Panx1 (in green) on platelets from WT or Panx1$^{-/-}$ mice. Platelets were counterstained with Evans Blue (in red). Negative control (Neg) included omission of first antibody. Scale bar represents 5 μm. **E**. Collagen-induced ATP release from human platelets ($N = 5$) after 7 min preincubation with α-Panx1EL or α-Panx1CT antibodies (white). **F**. Quantification of time to cessation of tail bleeding in WT mice treated for 7 min with α-Panx1EL or α-Panx1CT (white) antibodies ($N = 9$–10). **G**. Quantification of time to respiratory arrest in WT mice treated for 7 min with α-Panx1EL or α-Panx1CT antibodies (white) prior to VTE induction ($N = 10$). $^*P < 0.05$, $^{\S}P = 0.07$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

however be kept in mind that the hemostatic response was also affected in the Panx1-knockout mouse models or with a single high dose of the novel specific Panx1 channel blocker. Whether besides ATP-mediated P2X1 channel activation, other pathways are modified in Panx1-deficient platelets or whether the potential interference of different signaling cascades does not occur at lower concentrations of α-Panx1EL remains subject of future studies. Given the current lack of such drugs, research towards new targets that would optimally balance antithrombotic effects and hemorrhagic risk remains mandatory.

Author contributions

FM and BRK designed the research, analyzed and interpreted the data, and wrote the manuscript; FM, MJM, GP, AH, YE, SM performed experiments; BAI, PF, SM, ES provided scientific inputs and mice; all authors critically revised the manuscript and approved submission.

Declaration of competing interest

None to report for any of the authors.

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