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Combined In Vivo Depletion of GPVI and CLEC-2 Severely Compromises Hemostasis and Abrogates Arterial Thrombosis in Mice

Running title: Platelets deficient in GPVI and CLEC-2

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Objective – Platelet inhibition is a major strategy to prevent acute ischemic cardiovascular and cerebrovascular events which may, however, be associated with an increased bleeding risk. The (hem)ITAM-bearing platelet receptors, GPVI and CLEC-2, might be promising antithrombotic targets as they can be depleted from circulating platelets by antibody treatment leading to sustained antithrombotic protection but only moderately increased bleeding times in mice.

Approach and Results - We investigated whether both (hem)ITAM-bearing receptors can be targeted simultaneously, and what the in vivo consequences of such a combined therapeutic GPVI/CLEC-2 deficiency are. We demonstrate that isolated targeting of either GPVI or CLEC-2 in vivo does not affect expression or function of the respective other receptor. Moreover, simultaneous treatment with both antibodies resulted in the sustained loss of both GPVI and CLEC-2 while leaving other activation pathways intact. However, GPVI/CLEC-2-depleted mice displayed a dramatic hemostatic defect and profound impairment of arterial thrombus formation. Furthermore, a strongly diminished hemostatic response could also be reproduced in mice genetically lacking GPVI and CLEC-2.

Conclusion - These results demonstrate that GPVI and CLEC-2 can be simultaneously downregulated in platelets in vivo and reveal an unexpected functional redundancy of the two receptors in hemostasis and thrombosis. These findings may have important implications of the potential use of anti-GPVI and/or anti-CLEC-2 based agents in the prevention of thrombotic diseases.

Key Words: platelets, GPVI, CLEC-2, hemostasis, thrombosis

Introduction

At sites of vessel wall injury components of the extracellular matrix (ECM), most importantly collagens, are exposed to the flowing blood which triggers sudden platelet activation and platelet plug formation, followed by coagulant activity and the formation of fibrin-containing thrombi that occlude the site of injury. These events are crucial to prevent posttraumatic blood loss but they are also a major pathomechanism in arterial thrombosis.[1, 2] Glycoprotein VI (GPVI) is the central platelet activating collagen receptor and is non-covalently associated with the FcRγ-chain that carries an immunoreceptor tyrosine activation motif (ITAM). Binding of GPVI to exposed subendothelial collagens finally results in platelet activation and subsequent thrombus growth.[3] Patients[4] and mice[5-8] lacking GPVI display defective platelet responses to collagen but only mild bleeding tendencies making this receptor a potential target for effective and safe antithrombotic therapy.[9] We have previously shown that in vivo treatment of mice with anti-GPVI antibodies leads to downregulation of the receptor from the surface of circulating platelets by internalization and ectodomain shedding involving multiple proteases resulting in a GPVI knockout-like phenotype and long-term anti-thrombotic protection but only very moderate effects on normal hemostasis.[10, 11] A comparable antibody-mediated GPVI depletion has also been observed in platelets of autoimmune patients who had developed anti-GPVI antibodies[4] or in human platelets circulating in NOD/SCID mice.[12]

Another receptor that mediates strong platelet activation is CLEC-2, a C-type lectin-like type II transmembrane receptor that was identified as the receptor for the platelet activating snake venom rhodocytin.[13] Interestingly, CLEC-2 is a so-called hemITAM receptor containing only a single cytoplasmic YXXL motif that uses a similar signaling pathway as the GPVI/FcRγ-chain complex.[14] Upon CLEC-2 engagement, hemITAM phosphorylation of CLEC-2 is mediated by the tyrosine kinase Syk which is essential for signaling and downstream phosphorylation of effector proteins, including PLCγ2.[15] A developmental role for CLEC-2, which is the receptor for the lymphatic endothelial cell expressed protein podoplanin, has been described as the constitutive CLEC-2 knockout led to embryonic/neonatal lethality in mice caused by blood-

lymphatic misconnection and severe edema.[16-18] However, how platelets mediate vessel separation is at present unclear and still controversially discussed.[18-20]

Principally, CLEC-2 might become a target for antithrombotic agents, but the lethality of CLEC-2 knockout mice has made studies on the function of the receptor in hemostasis and thrombosis difficult.[17, 21] We have demonstrated that CLEC-2 can also be downregulated in platelets by in vivo administration of a monoclonal anti-CLEC-2 antibody (INU1). Such CLEC-2-depleted mice display reduced thrombus stability and are protected from vessel occlusion in thrombosis models but show only moderately increased bleeding times.[22] Shortly later, two studies reported partially conflicting results on the role of CLEC-2 in hemostasis and thrombosis using chimeric mice lacking CLEC-2 in the hematopoietic system (*Clec2*$^{-/-}$) suggesting a significant or no involvement of the receptor in these processes.[17, 21]

Here we investigated whether the simultaneous targeting and thus downregulation of GPVI and CLEC-2, which are the only (hem)ITAM coupled receptors in mouse platelets,[23] is possible and what the functional consequences of such a treatment are. We show that both receptors can be specifically downregulated simultaneously. Remarkably, loss of both (hem)ITAM receptors resulted in severely defective hemostasis and arterial thrombus formation, revealing partially redundant functions of GPVI and CLEC-2 in vivo.

Results

Independent and Simultaneous Downregulation of GPVI and CLEC-2 in vivo

Mice were injected intravenously with the anti-GPVI antibody JAQ1 (100 µg), the anti-CLEC-2 antibody INU1 (200 µg), or both antibodies in combination. While JAQ1 treatment induced a rapidly reversible thrombocytopenia, a more sustained thrombocytopenia was observed in mice treated with INU1 or JAQ1/INU1 with recovery to normal platelet counts on day 5-6 (Figure 1A). JAQ1 treatment induced the complete loss of GPVI but had no effect on CLEC-2 surface expression levels. Similarly, INU1 treatment induced the complete loss of CLEC-2 from the platelet surface but had no effect on GPVI expression (Figure 1B). Moreover, CRP-induced GPVI signaling in CLEC-2-depleted platelets was not affected and also, vice versa, CLEC-2 signaling induced by rhodocytin was unaltered in GPVI-depleted platelets (Figure 1C). Platelets from mice treated with JAQ1 *and* INU1 specifically lacked GPVI and CLEC-2 whereas expression of other surface proteins was not or only slightly (GPIX, integrin $\alpha 2\beta 1$) altered (Figure 1D). Slightly increased size of double deficient platelets was observed on days 5-7 which is in agreement with general observations made after antibody-induced thrombocytopenia (Figure 1D). Double-deficient platelets were specifically refractory to the GPVI and CLEC-2 agonists CRP, convulxin and rhodocytin, respectively (Figure 1E, integrin activation, left; P-selectin exposure, right). Only slightly decreased P-selectin exposure after thrombin stimulation was observed at early (Figure 1E) but not later time points (not shown), in line with previous observations made in JAQ1-treated mice.[24] Similarly, double-deficient platelets showed absent aggregation responses to GPVI- or CLEC-2 specific agonists, whereas the cells normally aggregated in response to other agonists (Supplemental Figure I). These data clearly show that targeting of one (hem)ITAM bearing receptor specifically downregulates its expression and activity on the platelet surface but does not influence the expression and signaling induced pathway of the other respective (hem)ITAM bearing receptor. Moreover, it is possible via simultaneous injection of both antibodies, JAQ1 and INU1, to completely shut off ITAM signaling in mouse platelets without affecting signaling by G protein-coupled receptors.

Severely Defective Hemostasis and Arterial Thrombus Formation in GPVI/CLEC-2 Double Depleted Mice

We have previously shown that JAQ1-treated mice display very mildly prolonged bleeding times and also INU1-treated mice show only moderately increased but generally more variable tail bleeding times in the filter paper model[10, 22] and this was confirmed in the current study (Supplemental Figure II). Remarkably, however, the depletion of both (hem)ITAM-bearing receptors lead to a virtually complete loss of hemostatic activity as evident by the lack of cessation of tail bleeding (Figure 2A). The same observation was made when the wound of the tail tip was immersed in 37°C prewarmed saline. Here, single-deficient mice displayed normal hemostatic function, whereas double-deficient mice displayed again a strong bleeding phenotype (Figure 2B). Importantly, however, we did not observe any signs of spontaneous bleeding in any of these animals. These data suggest that GPVI and CLEC-2 may have at least partially redundant roles in hemostasis but that their simultaneous loss does not induce spontaneous hemorrhage.

The effect of single and double receptor depletion on pathologic thrombus formation was studied by intravital fluorescence microscopy of ferric chloride-injured mesenteric arterioles.[7, 22] In control mice, small aggregate formation was observed at 7.8 ± 1.2 minutes after injury (Figure 2C, left) with complete vessel occlusion occurring at 16.4 ± 2.2 minutes (not shown, Supplemental video I). GPVI and CLEC-2 single-depleted mice showed similar kinetics of small aggregate formation whereas in most cases the vessels did not occlude (GPVI-depleted: 8/12; Supplemental video II; CLEC-2-depleted: 7/10; Supplemental video III, see also references[7, 22]). Remarkably, onset of small aggregate formation was significantly delayed in GPVI/CLEC-2-depleted mice (Figure 2C, left) and the maximal vessel stenosis reached within the 40 minutes observation period was strongly reduced compared to all other groups (Figure 2C, right). As a consequence, blood flow was maintained in all vessels (12/12, Supplemental video IV). Representative images from the experiment are shown (Figure 2C). These data demonstrate that the lack of both GPVI and CLEC-2 results in almost completely abolished thrombus formation suggesting partially redundant functions of

the two receptors *in vivo* and that their simultaneous targeting provides profound antithrombotic protection but also severely impairs normal hemostasis

Defective Hemostasis in CLEC-2 Depleted *Gp6*$^{-/-}$ Mice

The severe hemostatic defect in JAQ1/INU1-treated wild-type mice indicated that the "therapeutic" depletion of either receptor may induce bleeding in individuals genetically deficient in or expressing very low levels of the respective other receptor. To test this hypothesis directly, we studied platelet function in newly generated *Gp6*$^{-/-}$ mice (Supplemental Figure III) on day 5 after vehicle or INU1 treatment. As expected, *Gp6*$^{-/-}$ platelets were refractory to GPVI specific agonists as measured by flow cytometry and aggregometry whereas responses to other agonists were normal (not shown). In contrast, platelets from *Gp6*$^{-/-}$/INU1-treated mice lacked GPVI and CLEC-2 (Figure 3A) and were unresponsive towards CRP, convulxin, and rhodocytin, whereas all other tested activation pathways were unaffected (Figure 3B). Slightly increased GPIb expression levels were noted, which may be explained by the slightly increased platelet size. Similar to double-depleted mice (Figure 2A, B), CLEC-2-depleted *Gp6*$^{-/-}$ mice showed a severe hemostatic defect in both bleeding time assays (Filter paper: Figure 3C, Saline: Figure 3D) mirroring the JAQ1/INU1 antibody-induced double-deficiency.

Defective Hemostasis in Mice Genetically Deficient in Platelet GPVI and CLEC-2

To test the possibility that side effects of the antibody treatment contributed to the observed bleeding phenotype in receptor depleted animals, we generated mice genetically deficient in both receptors in platelets. As mice constitutively lacking CLEC-2 die perinatally,[17, 21] *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice specifically lacking CLEC-2 in megakaryocytes and platelets were used for analysis.[18] However, as previously described these mice are not healthy in that they display a pronounced defect in blood-lymph separation (Figure 4A)[16-18, 20] and other vascular defects which may influence the hemostatic system as indicated by a reduction in platelet count to

approximately 70% of control (Figure 4B). Platelets of *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice lacked CLEC-2 whereas all other tested surface receptors were normally expressed (Figure 4C). Consequently, rhodocytin-induced platelet activation was abolished in the mutant cells whereas responses to other agonists were fully intact (Figure 4D, integrin activation and P-selection exposure and Supplemental Figure IV: aggregometry). We have previously shown that CLEC-2 single-depleted mice display normal small aggregate formation in $FeCl_3$-injured mesenteric arterioles but were in most cases unable to fully occlude the vessels.[22] Similarly, *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice showed only slightly but not significantly delayed first appearance of small thrombi (Figure 4E, left) and vessel occlusion was in most of the animals delayed or absent (Figure 4E, right and Supplemental Videos V and VI). These results indicated that antibody-induced and genetic loss of platelet CLEC-2 provides comparable protection from occlusive thrombus formation.

We intercrossed *Gp6*$^{-/-}$ and *Clec-2*$^{fl/fl}$ mice and thereafter mated *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl}$ females with with *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ males to obtain double deficient animals. These breedings only yielded small litters (2-6 mice) and <35% *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice indicating increased embryonic or perinatal lethality. The surviving *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ animals displayed dramatically altered vascular structure and blood-filled lymphatics in the intestine and this phenotype was clearly more pronounced than in CLEC-2 single deficient mice (Supplemental Figure V). Flow cytometric analysis confirmed the absence of both receptors in the platelets of *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice which was associated with some minor changes in the expression pattern of other surface receptors and a moderately reduced platelet count similar to that observed in CLEC-2 single deficient mice (Figure 5A). As expected, the platelets of these mice showed a complete loss of (hem)ITAM signaling as revealed by measurement of αIIbβ3 activation and P-selection expression with GPVI or CLEC-2 specific agonists while leaving activation of these pathways by ADP, thromboxane and thrombin receptors intact (Figure 5B). In addition, we studied GPIb function in GPVI and CLEC-2 deficient platelets by two different assays, namely platelet spreading on a vWF coated matrix[25] and platelet adhesion on vWF under flow conditions.[26] In both cases, there was no significant difference

as compared to control indicating intact GPIb function in the mutant platelets (data not shown).

To test the effect of genetic GPVI/CLEC-2 double deficiency on hemostasis, tail bleeding times in mutant and control mice were assessed by the filter paper model (Figure 5C) and the saline model (Figure 5D). *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice showed in both models markedly prolonged bleeding times as compared to control or single-deficient mice confirming that GPVI and CLEC-2 have unexpected redundant roles in normal hemostasis. The bleeding time prolongation was less pronounced than in double-depleted mice, which may at least partially be explained by the vascular alterations and the reduced general state of health in these animals.

Attempts to study thrombus formation by intravital microscopy in $FeCl_3$-injured mesenteric arterioles as shown for GPVI/CLEC-2 depleted mice (which displayed no vessel separation defect [Supplemental Figure VI]), failed for *Gp6*$^{-/-}$*/Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice because of a dramatically altered vascular structure and blood-filled lymphatics in the intestine of these animals (Supplemental Figure V).

Discussion

In this study we have shown that the two major (hem)ITAM receptors, GPVI and CLEC-2, can be simultaneously depleted with high specificity in circulating platelets in vivo and that their combined loss results in a severe hemostatic defect and virtually abolished thrombus formation in mice. These findings reveal for the first time that GPVI and CLEC-2 have partially redundant functions in normal hemostasis and pathological thrombus formation and that their simultaneous targeting may be an effective, but not necessarily safe antithrombotic approach.

Both activatory receptors have been proposed as possible pharmacological targets for antithrombotic therapy because they can easily be immunodepleted from circulating platelets in vivo resulting in a knockout-like phenotype for the respective receptor for a prolonged period of time.[10, 22] Such a targeted downregulation of GPVI or CLEC-2 provides profound antithrombotic protection in different models of thrombosis while having only (very) moderate effects on normal hemostasis.[7, 10, 17, 21, 22] In this study, we could confirm these previous findings and show that the antibody induced loss of either GPVI or CLEC-2 does not affect expression or function of the respective other receptor. This appears to be different to the previously described phenomenon in human platelets in which cross-inhibition between GPVI and another ITAM-bearing receptor, FcγRIIa, which is only present on human but not mouse platelets, was observed.[23] Gardiner *et al.* showed[27] that FcγRIIa-ligation resulted in metalloproteinase-mediated ectodomain shedding of GPVI in vitro, demonstrating that signaling by one ITAM-bearing receptor not only influences its own expression and signaling but also causes effects on the other ITAM-bearing receptor. This suggests that this “trans-inhibition” effect of FcγRIIa and GPVI in human platelets in vitro occurs through a mechanism that is not operating in the regulation of CLEC-2 and GPVI in mouse platelets in vivo. Recently, a role for FcγRIIa as a functional conduit for αIIbβ3-mediated outside-in signaling and thus in controlling thrombosis was described in human platelets.[28]

The antibody-induced downregulation of GPVI occurs through two different pathways, namely internalization/degradation and more importantly metalloproteinase-dependent ectodomain shedding.[11, 29] Both processes require signaling through the FcRγ-chain ITAM[29] and can occur in circulating platelets and presumably also in megakaryocytes.[10] This GPVI immunodepletion appears to be very specific as GPVI-depleted and *Gp6*$^{-/-}$ mice display virtually identical defects in different thrombosis models and a comparable minor prolongation of tail bleeding times.[7] In contrast, much less is known about the mechanisms underlying the antibody-induced loss of CLEC-2 in platelets which is associated with a prolonged phase of marked thrombocytopenia.[22] It is currently not clear whether the induced loss of CLEC-2 can occur in circulating platelets in the periphery or also in megakaryocytes and whether it is mediated by ectodomain shedding, internalization or another, yet undefined mechanism. However, very similar to the loss of GPVI, CLEC-2 depletion is a surprisingly specific process leaving G protein-coupled receptor signaling pathways largely intact (Figure 1B, C and data not shown).[22] We have previously shown that CLEC-2 depleted mice display variable tail bleeding times when assessed in the filter paper model, which was also confirmed here (Supplemental Figure IIB).[22] In contrast to this, we have also reported that radiation chimeric mice lacking CLEC-2 in the hematopoietic system have unaltered tail bleeding times compared to wild-type controls when assessed using a version of the tail bleeding assay which monitors the time to cessation of bleeding without the use of filter paper.[21] Interestingly, we also found no prolongation of tail bleeding times in CLEC-2 depleted mice using a third version of this assay, namely monitoring bleeding into saline (Figure 2B), suggesting that the mechanisms contributing to hemostasis in the various bleeding time models may be partially different and that CLEC-2 depletion very well mirrors genetic loss of CLEC-2 in platelets. This is further corroborated by the observation that *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice show a very similar thrombus formation defect as CLEC-2 depleted mice as revealed by intravital microscopy using the ferric chloride injury model (Figure 4E and Supplemental Videos V and VI).[22] We tested whether the thrombus formation defect in CLEC-2 deficient mice could be due to a defect in fibrinogen binding. However, we found no

differences between CLEC-2 depleted and control platelets when assessing spreading on a fibrinogen coated surface, adhesion under flow on a fibrinogen matrix (rate: 1000 s^{-1}; data not shown)[30] or aggregation in response to ADP, thromboxane and thrombin receptor activation. Together, the data indicate that the targeted depletion of GPVI or CLEC-2 in circulating platelets can be induced in a highly specific manner and reproduces the phenotypes observed in mice with genetic deletions of these receptors.

The simultaneous injection of both antibodies, JAQ1 and INU1, resulted in a highly selective and complete loss of GPVI and CLEC-2 in platelets, respectively, while leaving other activation pathways largely intact. This demonstrates for the first time that it is possible to completely delete (hem)ITAM receptor function in circulating platelets in vivo. Further, the normal platelet count in these animals demonstrates that neither receptor is required for the steady level of platelet production. We found that the combined loss of GPVI and CLEC-2 resulted in markedly impaired hemostasis and a severe thrombus formation defect that by far exceeded that seen in GPVI- or CLEC-2 single depleted animals (Figure 2). Possible off-target effects of the antibody treatment do likely not explain this pronounced defect as it was fully reproduced in CLEC-2-depleted *Gp6*$^{-/-}$ mice and in *Gp6*$^{-/-}$/*Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice, although to a somewhat lesser extent in the latter which most likely could be due to the mixture of their blood and lymph (Figure 3C, D and 5C, D) and other vascular defects that may account for their increased embryonic/perinatal lethality and reduced general state of health at the adult stage. This assumption is also corroborated as neither the coagulation system (aPTT and PT, Supplemental Figure VII) was impaired nor relevant cytokine levels were released in double depleted mice (Supplemental Figure VIII) further excluding off-target effects of antibody treatment. Together, these findings demonstrate that GPVI and CLEC-2 have partially redundant functions in hemostasis and occlusive thrombus formation but the exact underlying mechanisms remain to be determined. For normal hemostasis, however, classic (hem)ITAM signaling downstream of the two receptors appears not to be essential as mice lacking Syk, a crucial proximal molecule in this signaling pathway, did not show such a bleeding defect.[31] Similarly, it has been reported that the Syk inhibitor, PRT060318, did not

affect hemostasis in mice.[32] Together, these findings point to functions of GPVI, CLEC-2 or both receptors in hemostasis and possibly also thrombosis independent of their classic signal transduction capacity. Based on this assumption, one may speculate that adhesive functions of these receptors and/or their ability to bind and activate putative counter receptors in platelets might account for this unexpected activity. However, currently no intravascular ligands for GPVI and/or CLEC-2 are known but based on our results we postulate that they may exist.

Taken together, we have demonstrated that antibody-mediated independent and simultaneous downregulation of the platelet activating proteins, GPVI and CLEC-2, is possible and revealed unexpected redundant functions of these two receptors in arteriolar thrombus formation but also, and more importantly, in normal hemostasis in mice. Although data obtained in mice cannot be directly extrapolated to the human system (which is further complicated by the possible role of a third ITAM receptor, FcγRIIa, which is absent in the mouse genome), these results indicate that anti-GPVI or anti-CLEC-2 treatment might bear the risk of uncontrolled bleeding in patients exhibiting defects in the respective other (hem)ITAM signaling pathway. Supporting data come from a very recent study that has appeared during revision of this manuscript showing that the ITAM receptors, GPVI and CLEC-2, are critical for vascular integrity in inflammatory processes.[33] Our results may have important implications for the development of anti-GPVI and/or anti-CLEC-2 based antithrombotic therapeutics.

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Disclosure

None.

Author Contributions

M. B. and F. M. performed experiments, analyzed data and contributed to writing of the manuscript. V. L., I. T., I. H., B. F., T. V., K. R., A. B. and M. Bö. performed experiments and analyzed data. SP. W. analyzed data and contributed to the writing of the manuscript. B. N. planned the project, analyzed data and contributed to writing of the manuscript.

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Significance

Platelet inhibition is a major strategy to prevent acute ischemic cardiovascular and cerebrovascular events, which may, however, be associated with an increased bleeding risk. The receptors, GPVI and CLEC-2, that are the only (hem)ITAM bearing receptors in mouse platelets, might be promising antithrombotic targets as they can be depleted from circulating platelets by antibody treatment leading to sustained antithrombotic protection but only moderately increased bleeding times in mice. Here, we found that combined loss of GPVI and CLEC-2, and thus (hem)ITAM signaling, resulted in markedly impaired hemostasis and a severe thrombus formation defect that by far exceeded that seen in GPVI- or CLEC-2 single depleted animals. These results indicate that anti-GPVI or anti-CLEC-2 treatment might bear the risk of uncontrolled bleeding in patients exhibiting defects in the respective other (hem)ITAM signaling pathway. Our results may have important implications for the development of anti-GPVI and/or anti-CLEC-2 based antithrombotic therapeutics.

Figure Legends

Figure 1. Analysis of mice deficient in GPVI and CLEC-2 upon antibody injection.

A, Mice were intravenously injected with 100 µg JAQ1 and/or 200 µg INU1 in sterile PBS and platelet counts were determined on a FACSCalibur at the indicated time points post injection. Results are mean ± SD in % of control animals (*n* = 5 mice per group, representative for 2 individual experiments). **B, D,** Flow cytometric analysis of surface protein expression five days post injection with the indicated antibodies. Platelets were stained for 15 minutes at room temperature with the indicated fluorophore-labeled antibodies and directly analyzed. Platelet count in number of platelets/µL. Platelet size is given as mean FSC and was determined by FSC characteristics. Results are mean fluorescence intensities (MFI) ± SD (*n* = 5, representative of at least three independent measurements). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **C, E,** Flow cytometric analysis of integrin αIIbβ3 activation (JON/A-PE) and degranulation-dependent P-selectin exposure on platelets on day 5 post injection. Washed

blood was incubated with the indicated agonists for 15 minutes and analyzed on a FACSCalibur. Results are mean ± SD (*n* = 5 mice per group, representative of three independent experiments). ***, P < 0.001. ADP: 10 µM; U46619: 3 µM; thrombin: 0.01 U/mL; rhodocytin: 1 µg/mL; CRP: 10 µg/mL; convulxin: 1 µg/mL. All experiments were performed on day 5-6 after antibody injection.

Figure 2. Determination of hemostatic function and pathological thrombus formation in GPVI/CLEC-2-depleted mice.

A, A 1 mm segment of the tail tip was cut and bleeding was determined to have ceased when no blood drop was observed on the filter paper. Each symbol represents one individual. **B,** A 1 mm segment of the tail tip was cut and the tail tip was immersed in saline. Each symbol represents one individual. Differences of bleeding times between wt, single GPVI-depleted and single CLEC-2-depleted mice are non-significant. **C,** Mesenteric arterioles were treated with 20% $FeCl_3$ and adhesion and thrombus formation of fluorescently labeled platelets were monitored by in vivo fluorescence microscopy. Statistical evaluation of the time to appearance of a first thrombus (left) and percentage of maximal vessel stenosis (right) are depicted. n ≥10. At most 2 arterioles of each mouse were analyzed. ** P< 0.01; *** P < 0.001. Vessel stenosis was determined by measuring maximal thrombus size divided by vessel diameter using the Metamorph software (Visitron). Representative images are shown. White asterisk indicates occluded vessel. All experiments were performed on day 5-6 after antibody injection.

Figure 3. Analysis of *Gp6*$^{-/-}$/CLEC-2-depleted mice.

A, Flow cytometric analysis of surface protein expression of *Gp6*$^{-/-}$ platelets five days post injection with the anti-CLEC-2 antibody INU1. Platelets were stained for 15 minutes at room temperature with the indicated fluorophore-labeled antibodies and directly analyzed. Platelet count in number of platelets/µL. Platelet size is given as mean FSC and was determined by FSC characteristics. Results are mean fluorescence intensities (MFI) ± SD (*n* = 5,

representative of at least three independent measurements). **, P < 0.01; ***, P < 0.001. **B,** Flow cytometric analysis of degranulation-dependent P-selectin exposure and integrin αIIbβ3 activation on platelets. Washed blood was incubated with the indicated agonists for 15 minutes at RT and analyzed on a FACSCalibur. Results are mean ± SD (*n* = 5 mice per group, representative of three individual experiments). ***, P < 0.001. ADP: 10 µM; U46619: 3 µM; thrombin: 0.01 U/mL; rhodocytin: 1 µg/mL; CRP: 10 µg/mL; convulxin: 1 µg/mL. **C,** A 1 mm segment of the tail tip was cut and bleeding was determined to have ceased when no blood drop was observed on the filter paper. Each symbol represents one individual. Differences of bleeding times between control, *Gp6*$^{-/-}$ mice and CLEC-2-depleted mice are non-significant. **D,** A 1 mm segment of the tail tip was cut and the tail tip was immersed in saline. Each symbol represents one individual. All experiments were performed on day 5-6 after antibody injection. Bleeding time of *Gp6*$^{-/-}$/CLEC-2-depleted mice is significantly prolonged compared to control and *Gp6*$^{-/-}$ mice ***, P < 0.001 and to CLEC-2-depleted mice **, P < 0.01. Bleeding time of CLEC-2-depleted mice is prolonged compared to control **, P < 0.01.

Figure 4. Analysis of megakaryocyte/platelet-specific CLEC-2 deficient mice.

A, Representative images of the intestine are shown. L= lymphatic vessel, A= arteriole, V= vein. **B,** Platelet count was determined by flow cytometric analysis. ***, P < 0.001. **C,** Flow cytometric analysis of surface protein expression. Platelets were stained for 15 minutes at room temperature with the indicated fluorophore-labeled antibodies and directly analyzed. Platelet size is given as mean FSC and was determined by FSC characteristics. Results are mean fluorescence intensities (MFI) ± SD (*n* = 4, representative of at least three independent measurements). ***, P < 0.001. **D,** Flow cytometric analysis of degranulation-dependent P-selectin exposure and integrin αIIbβ3 activation on platelets. Washed blood was incubated with the indicated agonists for 15 minutes at RT and analyzed on a FACSCalibur. Results are mean ± SD (*n* = 5 mice per group, representative of three individual experiments). *, P < 0.05; ***, P < 0.001. ADP [µM]; U46619: [µM]; thrombin: [U/mL]; rhodocytin: [µg/mL]; CRP:

[µg/mL]; convulxin: [µg/mL]. **E,** Mesenteric arterioles were treated with 20% $FeCl_3$ and adhesion and thrombus formation of fluorescently labeled platelets were monitored by in vivo fluorescence microscopy. Evaluation of the time to appearance of a first thrombus (left) and time to vessel occlusion (right) are depicted. n ≥10. Time of first appearance of thrombi is non-significant. Time to occlusion is *, P < 0.05.

Figure 5. Analysis of GPVI and CLEC-2 double mutant mice.

A, Flow cytometric analysis of surface protein expression of *Gp6*$^{-/-}$/ *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ platelets. Platelets were stained for 15 minutes at room temperature with the indicated fluorophore-labeled antibodies and directly analyzed. Platelet count in number of platelets/µL. Platelet size is given as mean FSC and was determined by FSC characteristics. Results are mean fluorescence intensities (MFI) ± SD (*n* = 5, representative of at least three independent measurements). *, P < 0.05; **, P < 0.01; ***, P < 0.001. **B,** Flow cytometric analysis of degranulation-dependent P-selectin exposure and integrin αIIbβ3 activation on platelets. Washed blood was incubated with the indicated agonists for 15 minutes at RT and analyzed on a FACSCalibur. Results are mean ± SD (*n* = 4 mice per group, representative of three individual experiments). **, P < 0.01; ***, P < 0.001. ADP [µM]; U46619: [µM]; thrombin: [U/mL]; rhodocytin: [µg/mL]; CRP: [µg/mL]; convulxin: [µg/mL]. **C,** A 1 mm segment of the tail tip was cut and bleeding was determined to have ceased when no blood drop was observed on the filter paper. Each symbol represents one individual. Fisher test: *Gp6*$^{-/-}$/ *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ vs. control: 0.0351; *Gp6*$^{-/-}$/ *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ vs. *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$: 0.0087. Other conditions non-significant. **D,** A 1 mm segment of the tail tip was cut and the tail tip was immersed in saline. Each symbol represents one individual. Bleeding time of *Gp6*$^{-/-}$/ *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice is prolonged compared to control and *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ *, P < 0.05.

MATERIALS AND METHODS

Mice

Male NMRI and C57BL/6JRj mice 3-6 weeks of age were obtained from Harlan (Borchen, Germany) or Janvier (Le Genest St. Isle, France). Animal studies were approved by the local authorities (Bezirksregierung Unterfranken). Mice were intravenously injected with 100 µg anti-GPVI (JAQ1) and/or with 200 µg anti-CLEC-2 (INU1) antibody. *Gp6*$^{-/-}$ mice were generated as described in the supplement. *Clec-2*$^{fl/fl}$ [1] and *Pf4-Cre*[2] mice were described earlier.

Reagents and Antibodies

The anesthetic drugs medetomidine (Pfizer), midazolam (Roche), fentanyl (Janssen-Cilag), and the antagonists atipamezol (Pfizer), flumazenil (Delta Select) and naloxon (Delta Select) were used according to the regulation of the local authorities. High-molecular-weight heparin (Ratiopharm), Apyrase Grade III, human fibrinogen, ADP (Sigma-Aldrich), prostacycline (PGI_2, Calbiochem), U-46619 (Enzo Life Sciences), thrombin (Roche), collagen (Kollagenreagens Horm; Nycomed), convulxin (Axxora), were purchased. Collagen-related peptide (CRP) was generated as previously described.[3] Rhodocytin was isolated as described.[4] JON/A-PE antibody against the activated form of integrin αIIbβ3 was from Emfret Analytics. All other antibodies were generated and modified in our laboratory as previously described.[5, 6]

Determination of Platelet Count, Size, Surface Protein Expression and Platelet Activation

To measure platelet size and surface protein expression, heparinized blood was diluted 1:20 and stained for 15 minutes 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 monoclonal antibodies at saturating concentrations for 15 minutes at 37°C and directly analyzed.

Tail Bleeding Time

Filter paper: Mice were anesthetized and a 1-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20-second intervals, without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Otherwise, experiments were stopped after 20 minutes.

Saline: Mice were anesthetized and 1 mm of the tail tip was cut off. Immediately, tails were immersed in 0.9% isotonic saline at 37°C. The time until stop of bleeding (no blood flow for longer than 1 minute) was determined. Otherwise, experiments were stopped after 10 minutes.

Intravital Microscopy of Thrombus Formation in $FeCl_3$-Injured Mesenteric Arterioles

Mice (15-18 g or 4-5 weeks old) were anesthetized, and the mesentery was exteriorized through a midline abdominal incision. Arterioles were visualized with a Zeiss Axiovert 200 inverted microscope (x10) equipped with a fluorescent lamp source, and a CoolSNAP-EZ camera (Visitron). Digital images were recorded and analyzed off-line using a Metavue software. Injury was induced by topical application of a 3-mm^2 filter paper saturated with $FeCl_3$ (20%). Adhesion and aggregation of fluorescently labeled platelets (i.v. injection of Dylight-488 conjugated anti-GPIX Ig derivative beforehand) in

arterioles were monitored for 40 minutes or until complete occlusion occurred (blood flow stopped for longer than 1 minute).[7]

Statistics

Results from at least 3 experiments per group are presented as mean ± SD. Differences between two groups were assessed by Welch's test, whereas differences between more than two groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett's T3 as post-hoc test using SPSS Statistics 20. The Fischer's exact test was applied to assess variance in occurrence of occlusion. *P*-values <0.05 were considered statistically significant.

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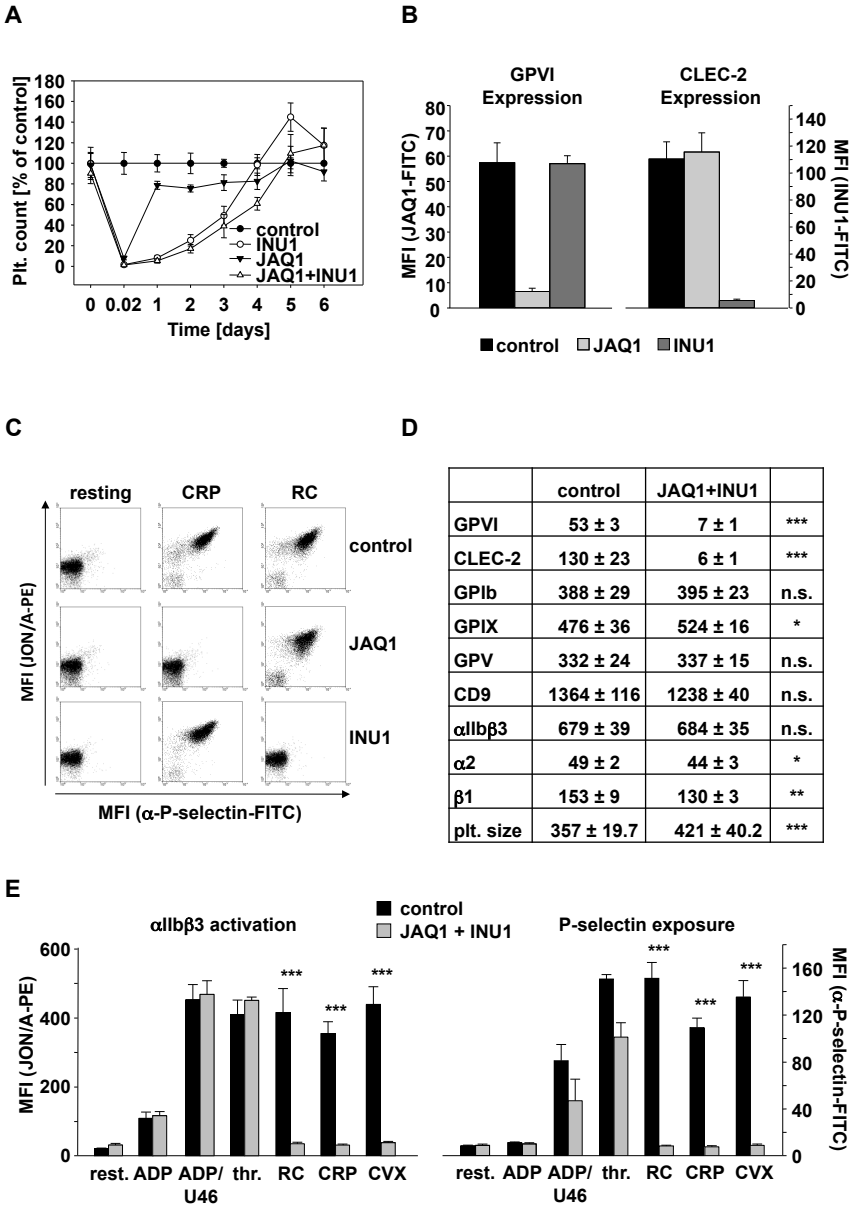


	control	JAQ1+INU1	
GPVI	53 ± 3	7 ± 1	***
CLEC-2	130 ± 23	6 ± 1	***
GPIb	388 ± 29	395 ± 23	n.s.
GPIX	476 ± 36	524 ± 16	*
GPV	332 ± 24	337 ± 15	n.s.
CD9	1364 ± 116	1238 ± 40	n.s.
αIIbβ3	679 ± 39	684 ± 35	n.s.
α2	49 ± 2	44 ± 3	*
β1	153 ± 9	130 ± 3	**
plt. size	357 ± 19.7	421 ± 40.2	***

Figure 1

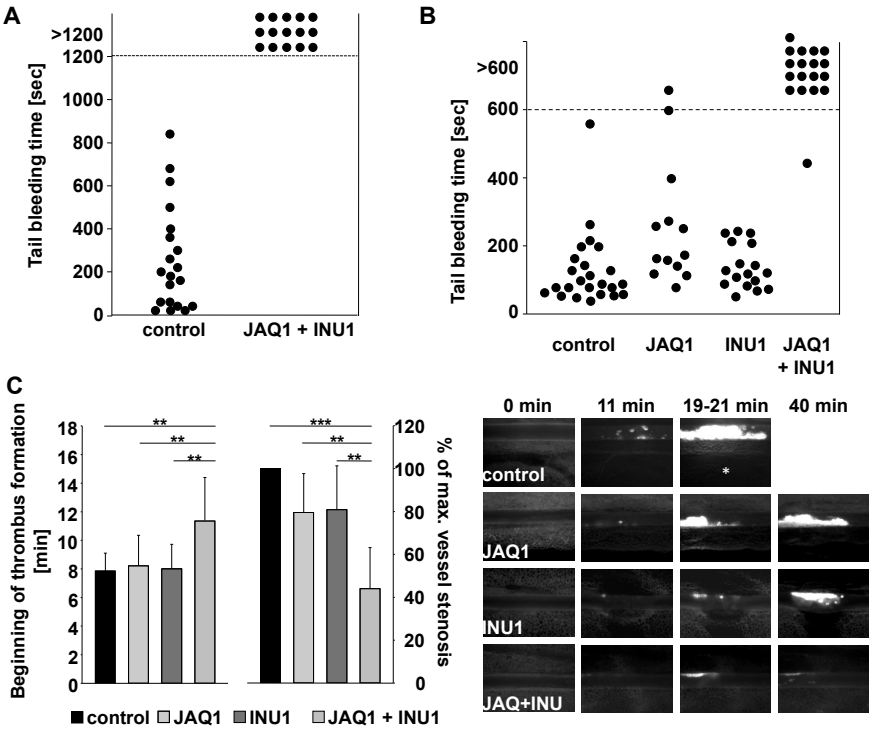
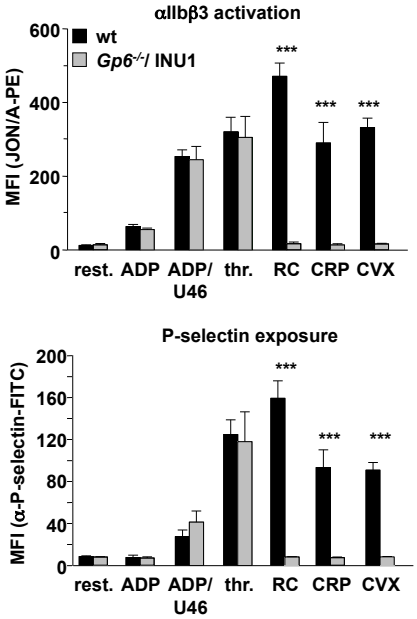

A
Tail bleeding time [sec]
>1200
1200
1000
800
600
400
200
0
control
JAQ1 + INU1
B
Tail bleeding time [sec]
>600
600
400
200
0
control
JAQ1
INU1
JAQ1 + INU1
C
Beginning of thrombus formation [min]
% of max. vessel stenosis
control
JAQ1
INU1
JAQ1 + INU1
0 min
11 min
19-21 min
40 min
control
JAQ1
INU1
JAQ+INU

Figure 2

A

	wt	*Gp6*$^{-/-}$/ INU1	
GPVI	38 ± 2	7 ± 0	***
CLEC-2	99 ± 5	10 ± 2	***
GPIb	481 ± 13	520 ± 20	**
GPIX	536 ± 13	529 ± 26	n.s.
GPV	343 ± 10	347 ± 24	n.s.
CD9	1654 ± 53	1561 ± 112	n.s.
αIIbβ3	716 ± 39	730 ± 101	n.s.
α2	78 ± 5	75 ± 6	n.s.
β1	156 ± 15	160 ± 10	n.s.
plt. size	326 ± 17	346 ± 21	n.s.
plt. count	682000 ± 59330	1187500 ± 62915	***

B



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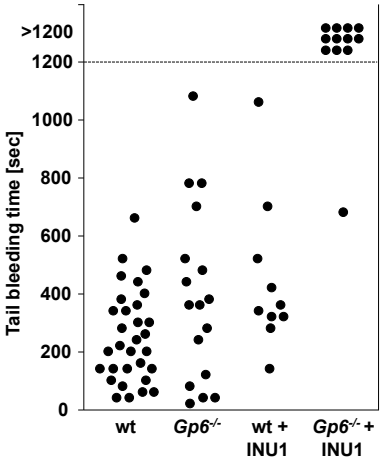

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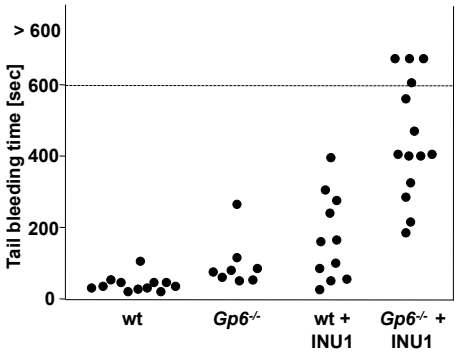
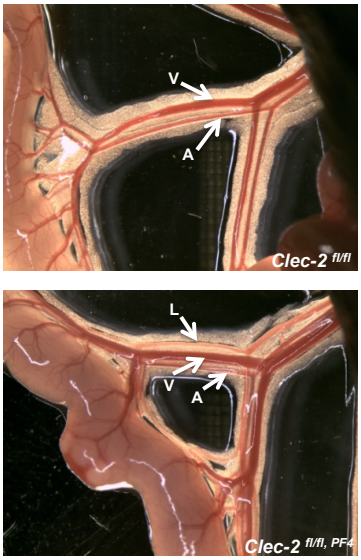


Figure 3

A



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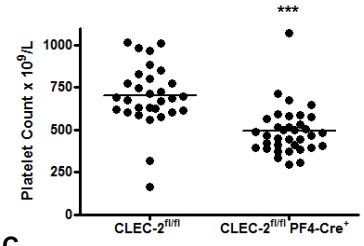
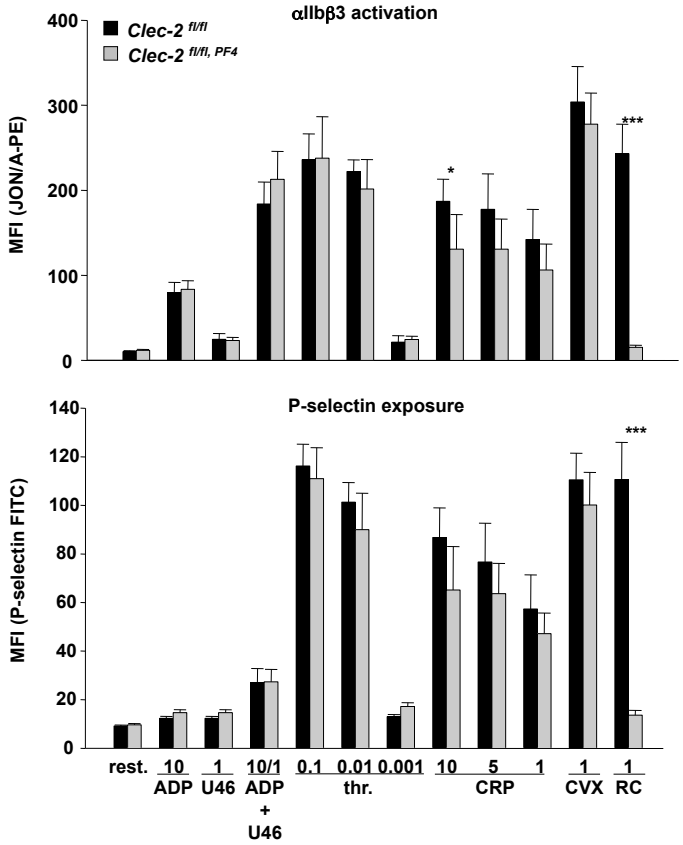


C

	Clec-2$^{fl/fl}$	*Clec-2*$^{fl/fl, PF4}$	
GPVI	49 ± 2	45 ± 7	n.s.
CLEC-2	143 ± 11	6 ± 0	***
GPIb	390 ± 26	377 ± 13	n.s.
GPIX	439 ± 14	437± 5	n.s.
GPV	318 ± 12	324 ± 6	n.s.
CD9	1392 ± 57	1377 ± 82	n.s.
αIIbβ3	488 ± 33	478 ± 32	n.s.
α2	50 ± 2	50 ± 2	n.s.
β1	153 ± 7	149 ± 2	n.s.
plt. size	289 ± 19	305 ± 12	n.s.

D



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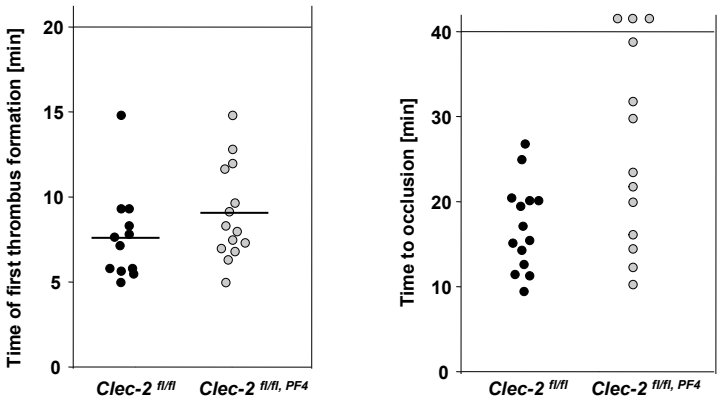
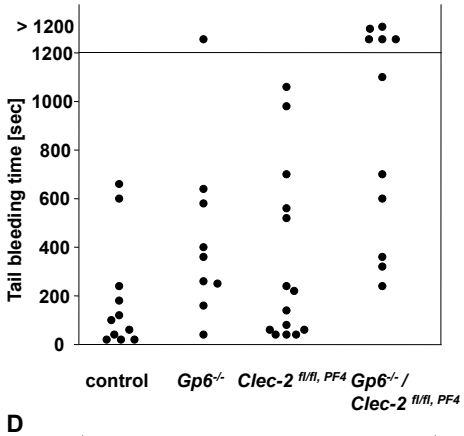


Figure 4

A

	control	*Gp6*$^{-/-}$/ *Clec-2* $^{fl/fl, PF4}$	
GPVI	65 ± 3	5 ± 0	***
CLEC-2	123 ± 9	6 ± 1	***
GPIb	427 ± 22	361 ± 16	**
GPIX	455 ± 12	414 ± 7	**
GPV	294 ± 22	269 ± 11	n.s.
CD9	1148 ± 63	1003 ± 71	*
αIIbβ3	438 ± 17	468 ± 29	n.s.
α2	53 ± 1	50 ± 3	n.s.
β1	127 ± 10	118 ± 3	n.s.
plt. size	352 ± 17	303 ± 9	**
plt. count	606250 ± 30000	468750 ± 55000	**

C



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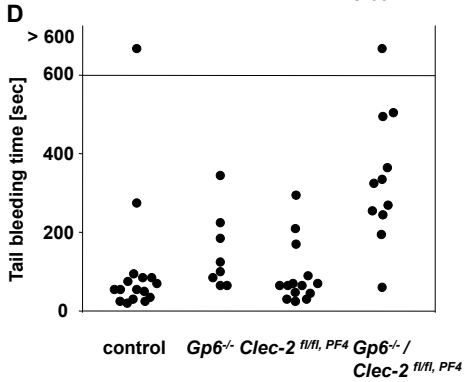

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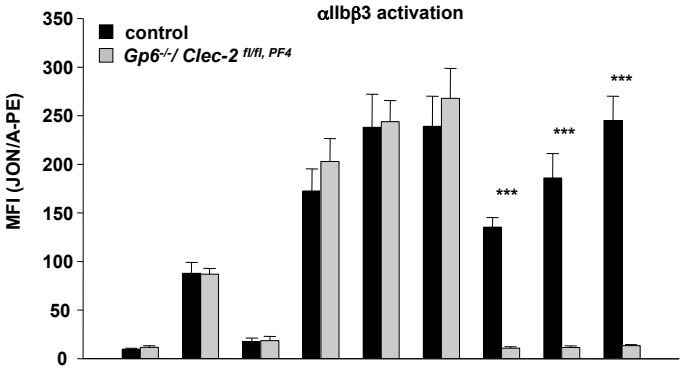


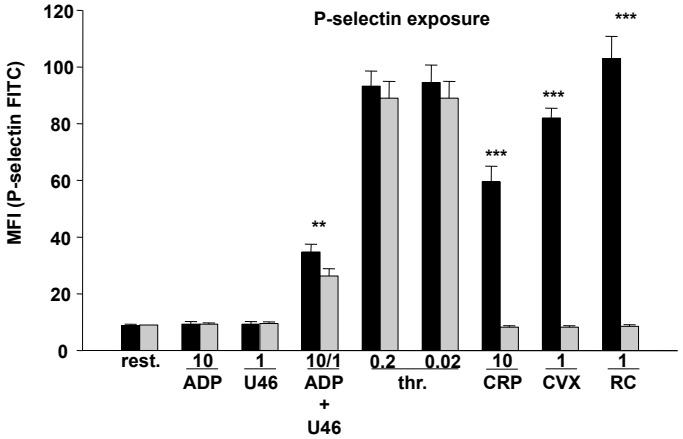


Figure 5

SUPPLEMENTAL MATERIAL

Combined In Vivo Depletion of GPVI and CLEC-2 Severely Compromises Hemostasis and Abrogates Arterial Thrombosis in Mice

Markus Bender, Frauke May, Viola Lorenz, Ina Thielmann, Ina Hagedorn, Brenda A. Finney, Timo Vögtle, Katharina Remer, Attila Braun, Michael Bösl, Steve P. Watson, Bernhard Nieswandt

Supplemental Methods

Generation of *Gp6*$^{-/-}$ Mice

BAC (bacterial artificial chromosome) clones containing the *Gp6* region were verified by PCR using specific primers against exon 2 and 3 and by physical mapping via Southern blotting (data not shown), and used as a PCR template to amplify the homologous arms for the generation of the targeting vector. Exon 2 and intron 2 were partially deleted and a marker and a neomycin resistance cassette were inserted and fused to exon 3. The targeting vector was electroporated into Sv129-derived embryonic stem (ES) cells to obtain homologous recombination. Successfully targeted ES cells were injected into C57BL/6 blastocysts. Germline transmission was obtained by backcrossing the resulting chimeric mice with C57BL/6 mice. *Gp6*$^{-/-}$ mice with a mixed Sv129/C57BL/6 background were used in this study.

Western Blotting

Proteins of lysed platelets were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. To monitor GPVI protein expression, after blocking the membrane was incubated with a HRP-labeled anti-GPVI (JAQ1) antibody and enhanced chemoluminiscence (ECL) detection substrate (MoBiTec) was used for visualization.

Aggregometry

Washed platelets (200 µL with 0.5 × 106 platelets/µL) were activated with the indicated agonists in the presence of 70 µg/mL fibrinogen and light transmission was recorded on a four-channel aggregometer (APACT, Laborgeräte und Analysensysteme, Hamburg) over 10 minutes and was expressed as arbitrary units with the light transmission of the buffer set at 100%.

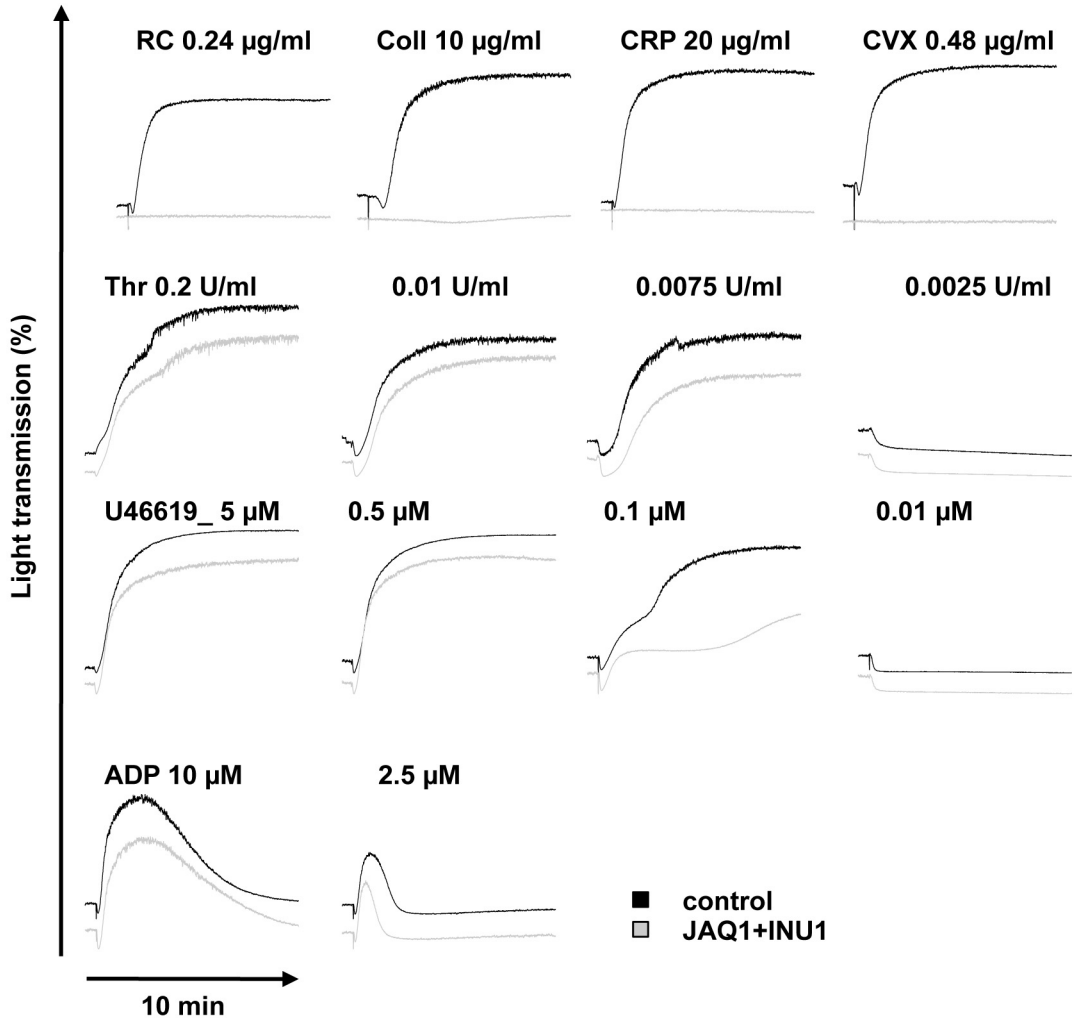
Cytokine ELISA

Plasma samples were assayed for levels of TNFα, IL-6, IFNγ and IL-1α by ELISA (Biolegend, Fell, Germany) according to the manufacturer's instructions. Samples were diluted 1:5 to 1:500 and analyzed using Multiskan EX plate reader with Ascent Software (Thermo Scientific, Dreieich, Germany).

Quantification of aPTT and Prothrombin Time

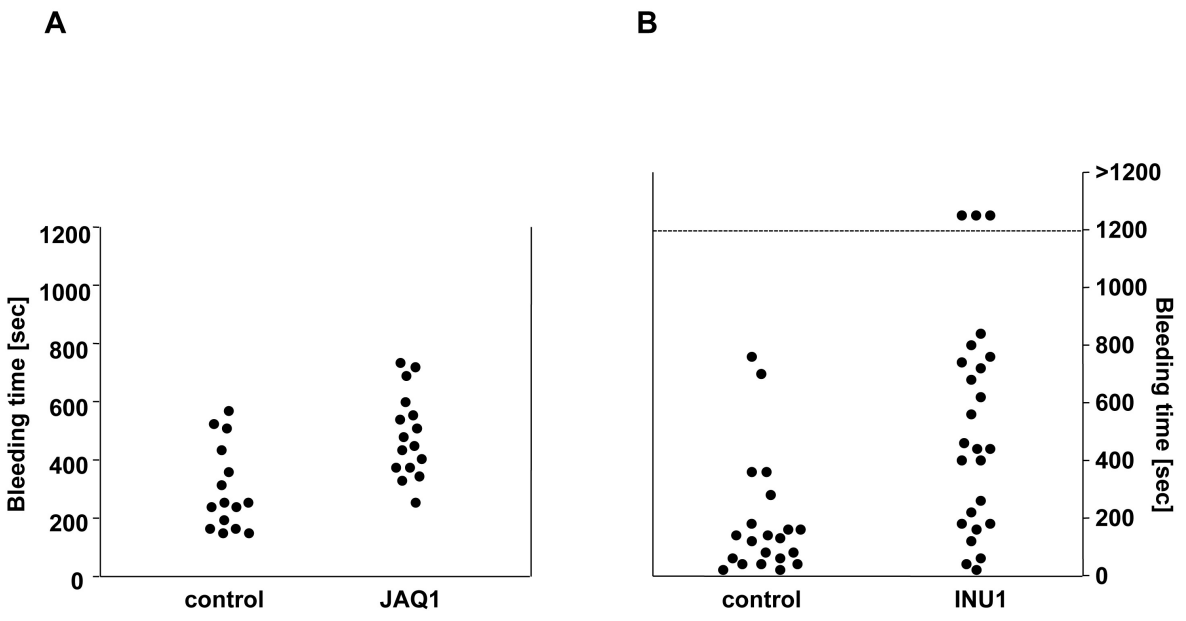
aPTT and prothrombin time (PT) of mouse plasma was determined by standard methods (Siemens Healthcare, Eschborn, Germany) in cooperation with the Zentrallabor University Hospital Wuerzburg.

Supplemental Figures



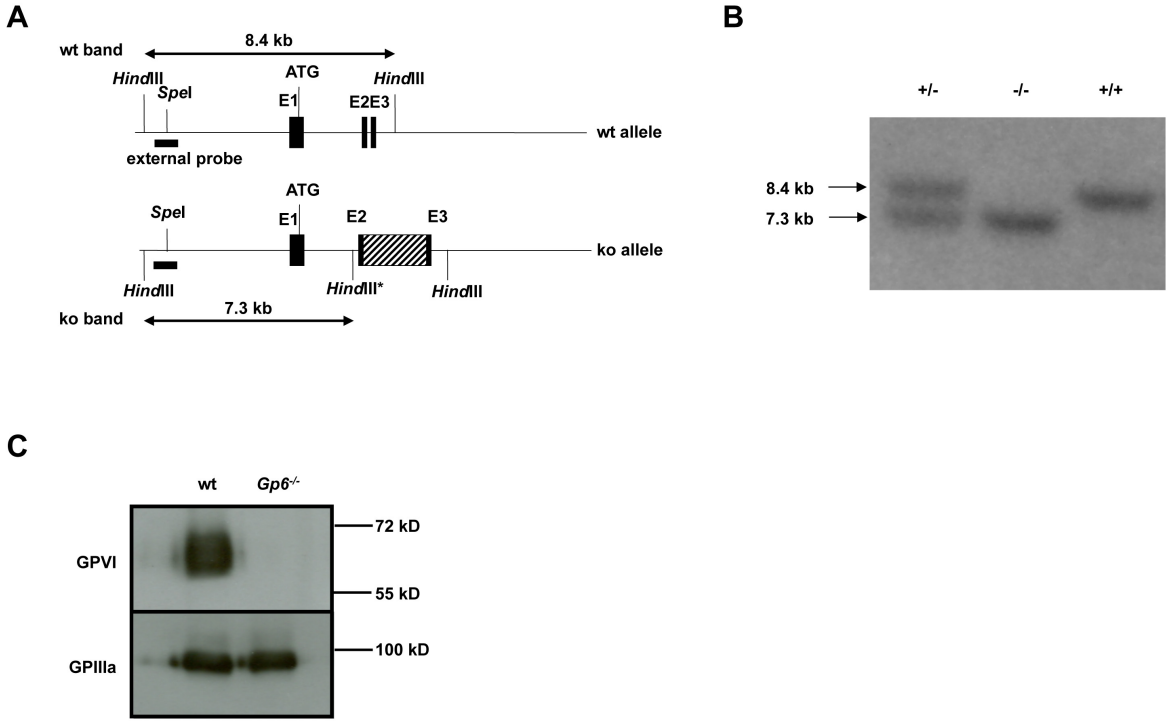
Supplemental Figure I. Representative aggregation curves of GPVI/CLEC-2 depleted washed platelets.

Aggregation studies were performed in buffer containing 70 µg/mL human fibrinogen. Thrombin-induced platelet aggregation was performed in the absence of human fibrinogen. ADP-induced platelet aggregation was performed with platelet-rich plasma. Representative curves of at least three independent measurements are shown. Indicated platelet agonist was added after 23 seconds.



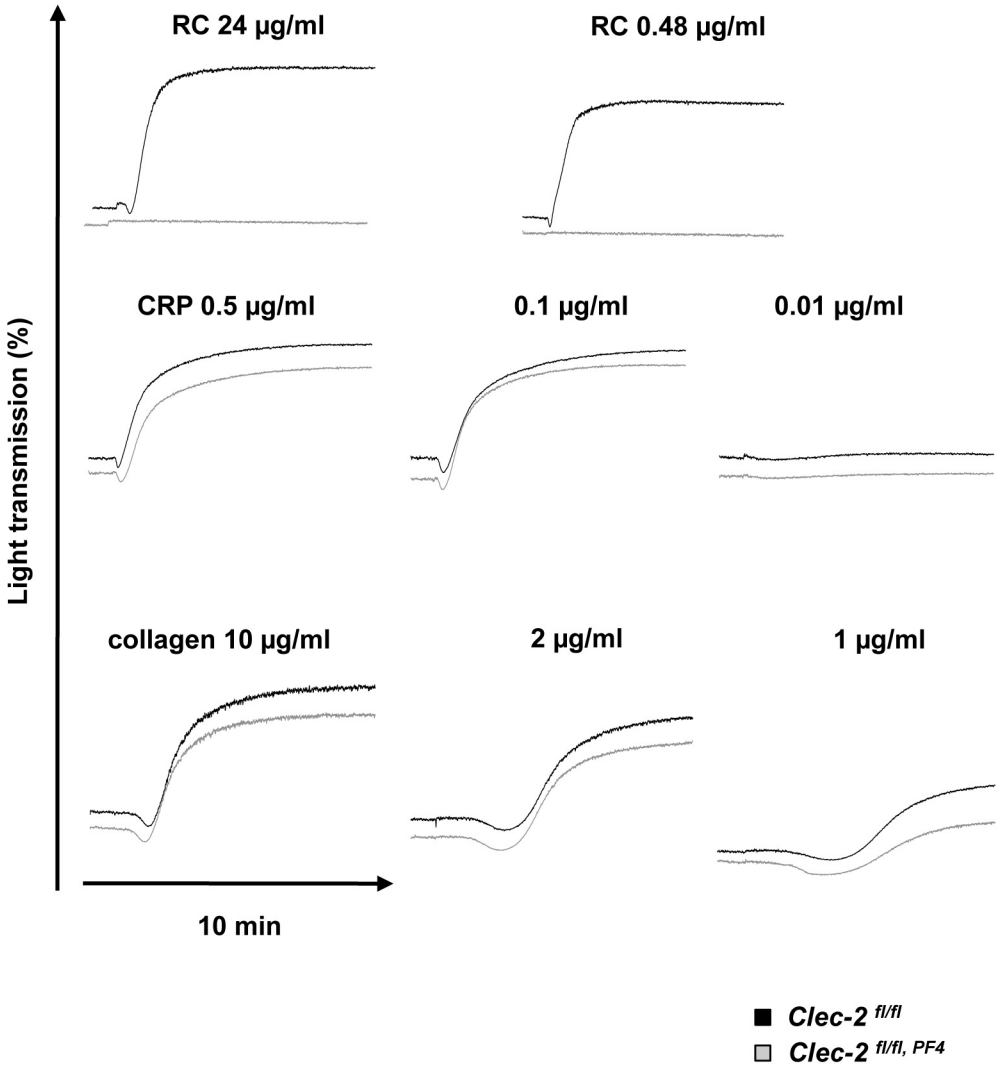
Supplemental Figure II. Measurement of tail bleeding times in GPVI and CLEC-2 single deficient mice.

Tail bleeding times were determined in **A,** GPVI-deficient and **B,** CLEC-2-deficient mice 5-7 days after either JAQ1 or INU1 antibody injection, respectively. A 1 mm segment of the tail tip was ablated and bleeding was determined to have ceased when no blood drop was observed on the filter paper.



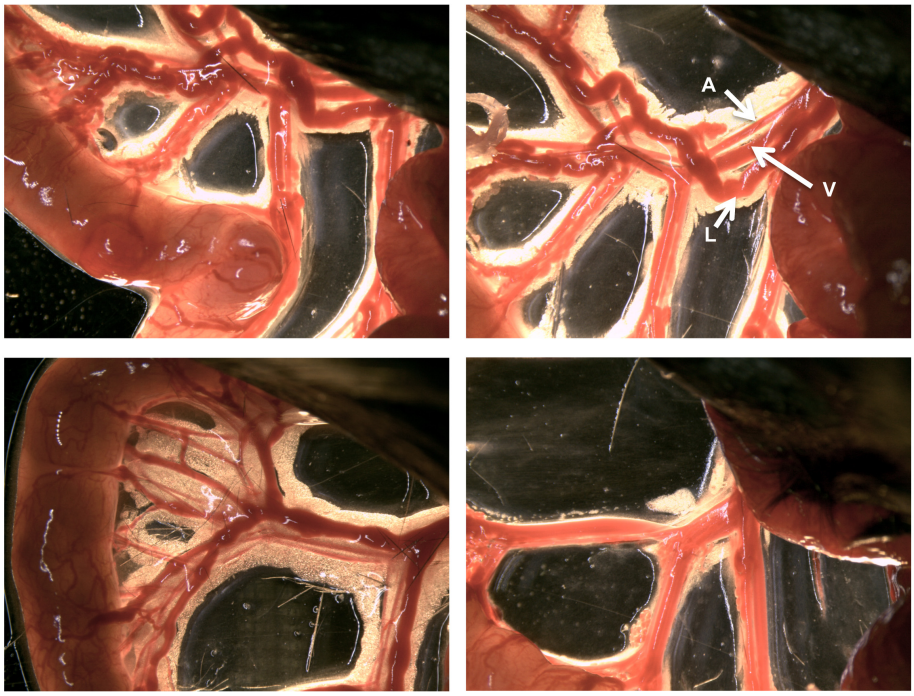
Supplemental Figure III. Generation of *Gp6*$^{-/-}$ mice.

A, The scheme depicts detection of *Gp6* wild-type (wt) and targeted (ko) bands. The external probe (black horizontal bar) recognizes a sequence upstream of exon 1 (E1). Exons are represented as black vertical bars. The wild-type band between two *Hind*III sites is 8.4 kb and the targeted band is 7.3 kb. Striped black box: neomycin resistance gene. **B,** Southern blot analysis from tail DNA of wild-type (+/+), targeted heterozygous (+/-) and targeted homozygous (-/-)*Gp6* mice. **C,** Western blot of platelet lysates from control and *Gp6*$^{-/-}$ mice. Whole platelet proteins were separated by SDS-PAGE and immunoblotted with an anti-GPVI (JAQ1) antibody. GPIIIa was used as a loading control.



Supplemental Figure IV. Representative aggregation curves of washed platelets from *Clec-2* $^{fl/fl,\ Pf4\text{-}Cre}$ mice.

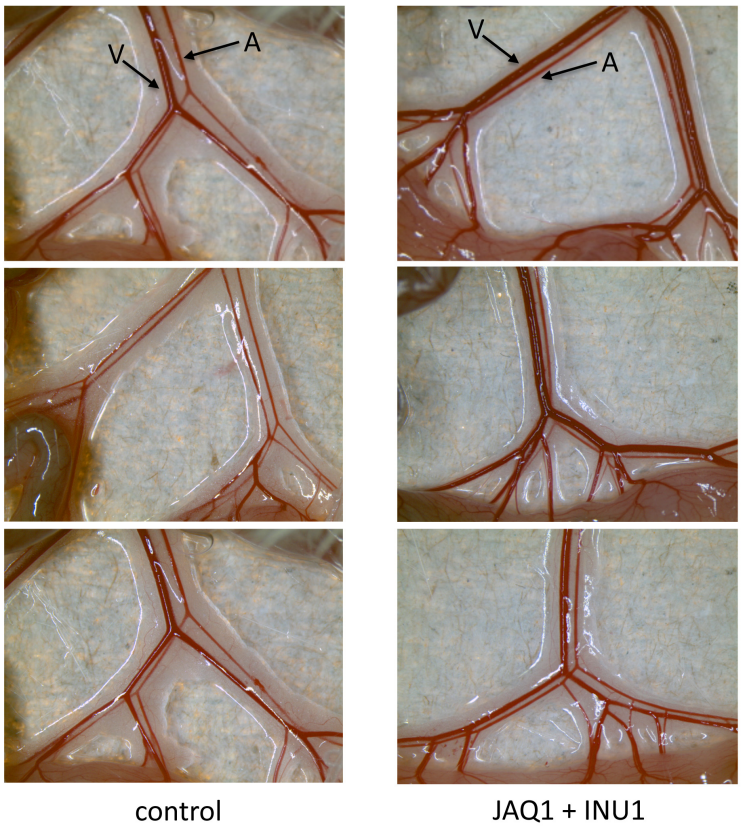
Representative curves of at least three independent measurements are shown. Indicated platelet agonist was added after 23 seconds.



Gp6 $^{-/-}$ / *Clec-2* $^{fl/fl,\ Pf4\text{-}Cre}$

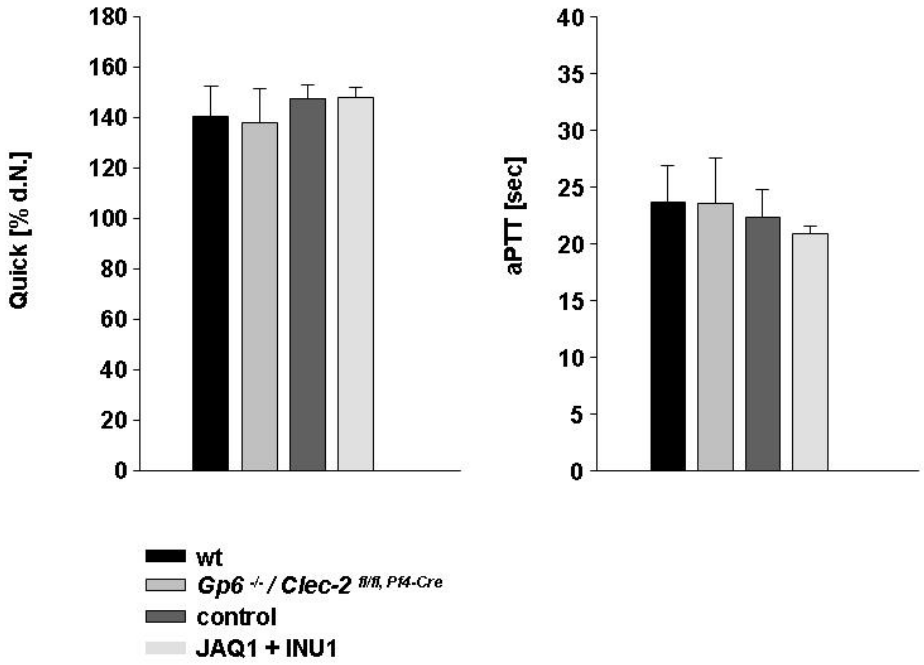
Supplemental Figure V. Representative images of the intestine of *Gp6*$^{-/-}$/*Clec-2* $^{fl/fl,\ Pf4\text{-}Cre}$ mice.

Double mutant animals display dramatically altered vascular structure and blood-filled lymphatics in the intestine. V: vein; A: artery; L: lymphatic vessel.

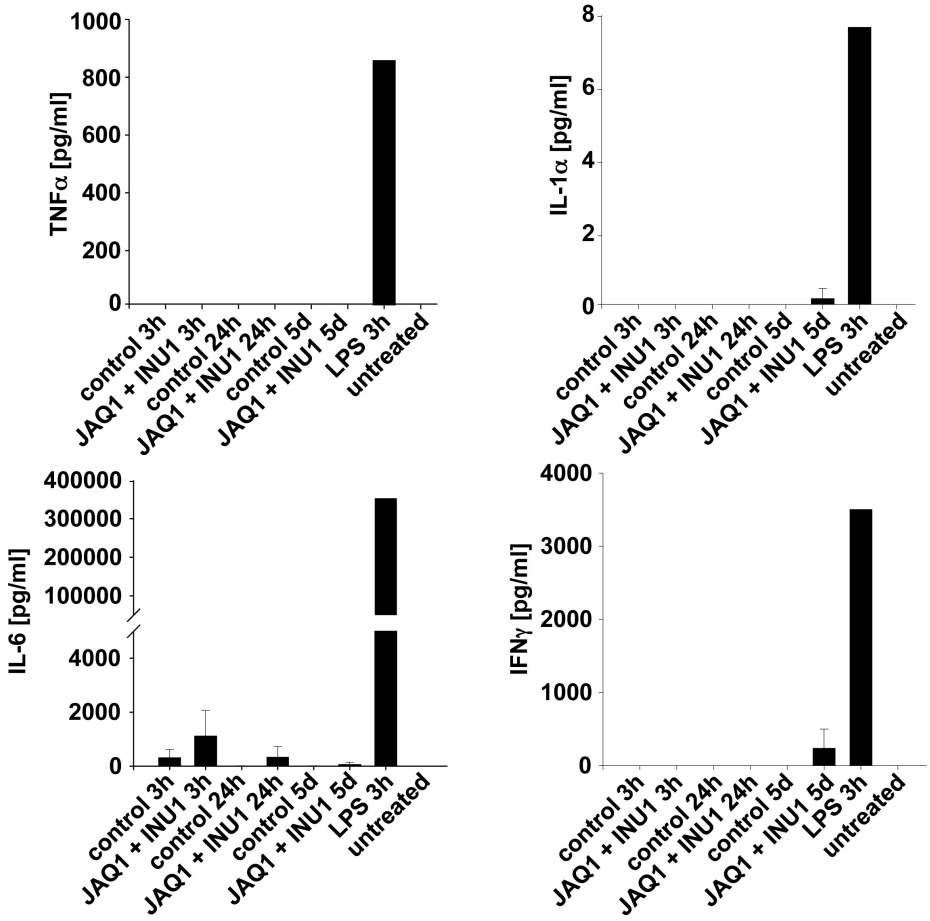


Supplemental Figure VI. Representative images of the intestine of JAQ1/INU1 treated mice.

Double depleted animals display normal vascular structure in the intestine. V: vein; A: artery.



Supplemental Figure VII. Determination of aPTT and PT in JAQ1 / INU1 treated and *Gp6*$^{-/-}$ */Clec-2* $^{fl/fl,\ Pf4\text{-}Cre}$ mice.



Supplemental Figure VIII. Plasma samples from JAQ1/INU1 treated mice were assayed for levels of TNFα, IL1α, IL6, and IFNγ by ELISA.

Only negligible or no cytokine levels were detectable.

h: hours; d: days; LPS: lipopolysaccharide as positive control.

Supplemental Videos. Time Lapse Video of *In Vivo* Thrombus Formation in Mice.

Mesenteric arterioles of control mice were exteriorized and endothelial injury was induced by application of $FeCl_3$. Platelets were labeled *in vivo* with a Dylight-488–coupled anti-GPIX Ig derivative, and platelet adhesion and thrombus formation was monitored in real time, and recorded on an inverted fluorescent microscope (Zeiss Axiovert 200) using a CoolSNAP-EZ camera (Visitron; exposure time: 700 ms). Representative video of at least 8 arteries per group, 1 second video corresponds to 1 minute recording time. Maximal observation time: 40 minutes.

Video I: A stable occlusive thrombus was formed after ~19 minutes in control mice.

Video II: In GPVI-depleted mice (day 5 post injection), large thrombi were formed which continuously embolized due to the lack of GPVI-collagen-interaction.

Video III: In CLEC-2-depleted mice (day 5 post injection), formed thrombi were completely instable, little fragments embolized and individual platelet released from the surface of growing thrombi.

Video IV: Beginning of platelet aggregate formation of JAQ1/INU1-treated mice (day 5 post injection) is clearly delayed. Formed thrombi remain considerably reduced in size and do not occlude the vessel.

Video V: A stable occlusive thrombus was formed after ~23 minutes in *Clec-2*$^{fl/fl}$ mice.

Video VI: Formed thrombi in mesenteric arterioles of *Clec-2*$^{fl/fl,\ Pf4\text{-}Cre}$ mice were completely instable, little fragments embolized and individual platelet released from the surface of growing thrombi.