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Collagen receptor glycoprotein VI promotes platelet-mediated aggregation of β-amyloid

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Abstract

Cerebral amyloid angiopathy (CAA) and β-amyloid (Aβ) deposition in the brain parenchyma are hallmarks of Alzheimer's disease (AD). We previously reported that platelets contribute to Aβ aggregation in cerebral vessels by secreting clusterin in response to the binding of Aβ40 to the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ [also known as glycoprotein IIb/IIIa (GPIIb/IIIa)]. Here, we investigated the contribution of glycoprotein VI (GPVI), a cardiovascular disease-associated collagen receptor, in platelet-induced amyloid aggregation. Using platelets isolated from GPVI-wildtype and GPVI-deficient human donors and mice, we found that Aβ40 bound to GPVI and induced tyrosine phosphorylation on target proteins that mediated the release of ATP and fibrinogen, resulting in platelet aggregation. Binding of Aβ40 to integrin $\alpha_{IIb}\beta_3$ (as previously reported), fibrinogen and GPVI led to the formation of amyloid clusters at the platelet surface. Consequently, pharmacological blockade of integrin $\alpha_{IIb}\beta_3$ or genetic loss of GPVI reduced the phosphorylation of LAT and amyloid fibril formation in cultured platelets and decreased the

adhesion of Aβ-activated platelets to the injured carotid artery in mice. Blocking GPVI-mediated signaling non-selectively with losartan decreased platelet activation, ATP and fibrinogen release, and Aβ40-stimulated platelet aggregation, although it did not reduce the formation of amyloid aggregates. Our findings indicate that Aβ40 binds to GPVI as well as integrin $\alpha_{IIb}\beta_3$ to promote platelet-mediated amyloid aggregation and, hence, blocking these pathways may therapeutically reduce amyloid plaque formation in cerebral vessels and the brain parenchyma of patients.

INTRODUCTION

In 2015, there were more than 47 million people living with dementia worldwide. With increasing age and the lack of effective therapeutic strategies, this number is projected to rapidly increase, reaching 135 million people by 2050 (1, 2). Alzheimer's disease (AD) is the most frequent cause of dementia, accounting for 60 % of dementia cases (3). The pathological hallmarks of AD are elevated misfolding, oligomerization and aggregation of β-amyloid (Aβ) peptides in brain parenchyma and in the cerebral vessels, known as cerebral amyloid angiopathy (CAA), and accumulation of intracellular neurofibrillary tangles (NFTs) in neurons (4, 5). The consequences are neurodegeneration with synaptic and neuronal loss leading to brain atrophy (6, 7).

Several studies indicate that vascular damage and dysfunction, including reduction of cerebral blood flow (CBF), cerebral amyloid angiopathy and blood-brain barrier (BBB) disturbances, contribute to the onset and progression of AD (8). Vascular risk factors such as atherosclerosis, stroke, hypertension and diabetes lead to vascular damage and are associated with AD. However, whether the processes in the vasculature initiate the pathologic process of Aβ aggregation is still uncertain. Identifying the mechanisms underlying vascular pathophysiology that contribute to neurodegeneration in AD will help identify novel therapeutic targets.

Besides the role of platelets in thrombus formation during hemostasis, it is becoming clear that platelets play a crucial role in a number of other processes within the vasculature such as angiogenesis, inflammation and cancer (9-11). Moreover, alterations in platelet function are also observed in diverse neurological diseases such as Parkinson disease, schizophrenia, autism and AD (12-15). A higher baseline expression of platelet activation biomarkers was measured in AD patients (16) and the analysis of the Alzheimer mouse model APP23 showed these mice have a pro-thrombotic phenotype (17, 18). Moreover, APP23 mice develop CAA and exhibit platelet accumulation at vascular plaques leading to the reduction of cerebral blood flow and probably to occlusion of cerebral vessels (18). The ability of platelets to modulate soluble, synthetic Aβ40 into fibrillar Aβ in vitro indicates a direct impact of platelets in the aggregation property of Aβ40 peptides (18-20). Previously we have demonstrated platelets contribute to amyloid-β aggregation through binding of Aβ40 to the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ leading to outside-in signaling in platelets (19, 20) and inhibition of integrin $\alpha_{IIb}\beta_3$ on the surface of platelets prevents the aggregation of Aβ40 in cultured cells (19). An important indication of the involvement of platelets in Aβ aggregation in vivo was shown by the treatment of APP23 mice with the antiplatelet agent clopidogrel, a $P2Y_{12}$ antagonist. Clopidogrel reduced the incidence of CAA with less adherent platelets at vascular Aβ deposits in transgenic AD mice

(19). Here, we investigated the involvement of other receptors on the surface of platelets and uncovered a critical role for the collagen receptor glycoprotein VI (GPVI) in platelet-mediated aggregation of Aβ.

RESULTS

Phosphorylation of tyrosine residues in LAT and other proteins by GPVI and integrin $\alpha_{IIb}\beta_3$ in response to Aβ40 stimulation

Binding of collagen to GPVI leads to a series of downstream signals in platelets, resulting in phosphorylation and activation of various signaling proteins, including the adaptor protein LAT (linker of activated T cells) (21). The stimulation of human platelets with soluble Aβ40 induced a similar pattern of tyrosine phosphorylation compared to the stimulation of platelets with CRP as shown by Western blot analysis (Fig. 1A). Aβ40 also induced the phosphorylation of LAT (Fig. 1B) and in a time-dependent manner, with a maximum abundance detected at 90 s of incubation (fig. S1). Previously, we demonstrated that binding of Aβ40 to integrin $\alpha_{IIb}\beta_3$ induces integrin outside-in signaling. To exclude this effect, we performed studies in the presence of human blocking integrin $\alpha_{IIb}\beta_3$ antibody Abciximab. Blocking of integrin $\alpha_{IIb}\beta_3$ decreased the phosphorylation of tyrosine (Fig. 1A) and LAT (Fig. 1B) induced by Aβ40. In addition, we used platelets from patients who lack the GPVI receptor. Human GPVI-deficient platelets did not show phosphorylation of LAT neither after stimulation with collagen nor by Aβ40 stimulation (Fig. 1C). However, Aβ40 induced phosphorylation of LAT was higher than upon activation with low concentration of collagen. Moreover, we studied the effect of Aβ40 in mouse platelets deficient in GPVI. Compared to wild type platelets, GPVI-deficient platelets failed to induce phosphorylation of LAT both upon CRP and Aβ40 activation (Fig. 1D). These results indicate the ability of Aβ40 to activate GPVI in human and mouse platelets.

Reduced ATP release of GPVI-deficient platelets in response to Aβ40

Activation of GPVI through collagen induces platelet activation leading to secretion of granules, inside-out signaling of integrin $\alpha_{IIb}\beta_3$ and platelet aggregation (22). To study the consequence of GPVI activation through Aβ40, we measured the release of ATP upon Aβ40 stimulation in human platelets. Previous studies showed that losartan inhibits collagen

induced platelet aggregation through GPVI (23, 24). Therefore, we analyzed the effects of losartan treatment on Aβ40 induced platelet stimulation. Aβ40 induced the release of ATP, however the amount of ATP was lower compared to CRP stimulation of platelets (Fig. 2A and B). To test whether Aβ40 induced ATP release is altered by losartan, platelets were pre-incubated with losartan. The release of ATP was reduced by losartan following stimulation of platelets with either CRP or Aβ40 (Fig. 2, A and B). WT and GPVI-knockout mice were analyzed to confirm that Aβ40 induces a release of ATP release via GPVI. The release of ATP in response to CRP or Aβ40 was significantly reduced using GPVI deficient platelets compared to wild type controls. Blocking of integrin $\alpha_{IIb}\beta_3$ using Leo.H4 antibody in WT platelets resulted in significantly reduced ATP release as well (Fig. 2C). Thus, Aβ40 induced release of ATP is mediated by GPVI and integrin $\alpha_{IIb}\beta_3$. Therefore, blocking of integrin $\alpha_{IIb}\beta_3$ in WT platelets reduced ATP release to resting levels (Fig. 2C).

Strongly reduced aggregation of GPVI deficient platelets in response to Aβ40

Next we analyzed platelet aggregation after Aβ40 stimulation. Aβ40 induced platelet aggregation that was comparable to that induced by CRP (Fig. 3A). Additionally, we analyzed the effect of losartan on Aβ40-induced platelet aggregation (Fig. 3A and B). In agreement with reported data, losartan significantly inhibited CRP- and Aβ40-induced platelet aggregation (Fig. 3A and B). To confirm the role of GPVI in Aβ40induced platelet aggregation, we used platelets from patients with GPVI deficiency. As expected, these platelets showed no platelet aggregation in response to Aβ40 compared to platelets from healthy controls (Fig. 3C and D). Moreover, we analyzed platelets from WT and GPVI-deficient mice. The aggregation response of WT mouse platelets with Aβ40 was comparable to CRP-induced platelet aggregation (Fig. 3E and F). As expected, GPVI-deficient mouse platelets showed no aggregation upon CRP stimulation. Platelet aggregation upon Aβ40 stimulation was reduced in GPVI-deficient platelets compared to WT platelets (Fig. 3F). In contrast to CRP stimulation, we still measured a slight platelet aggregation of GPVI-deficient platelets in response to Aβ40, suggesting that Aβ40 can induce platelet aggregation without GPVI. These results demonstrated that activation of GPVI by Aβ40 binding induced platelet aggregation.

Decreased amyloid aggregate formation by GPVI inhibition or genetic deletion in vitro

In our previous study, we showed that platelets are able to modulate soluble Aβ40 to fibrillar Aβ aggregates while blocking of integrin $\alpha_{IIb}\beta_3$ on the surface of platelets prevents Aβ aggregate formation (19). To investigate a role of GPVI in platelet mediated Aβ aggregate formation, human platelets were pretreated with losartan and incubated with soluble, synthetic Aβ40 for 3

days. The formation of fibrillar Aβ aggregates were analyzed by congo red staining. Although Aβ40 induced platelet aggregation and ATP release were reduced in the presence of losartan, we did not observe alterations in Aβ aggregation in platelet cell culture (Fig. 4A). Neither daily addition nor different concentrations of losartan were able to reduce fibrillar Aβ aggregate formation (fig. S2A to C).

Because losartan is not a specific GPVI inhibitor, mouse platelet experiments were performed where GPVI was blocked by antibody treatment with JAQ1. In addition, the antibody Leo.H4 was used to block integrin $\alpha_{IIb}\beta_3$ to confirm the essential role of integrin $\alpha_{IIb}\beta_3$ in Aβ aggregate formation. The formation of Aβ aggregates was completely inhibited by blocking of integrin $\alpha_{IIb}\beta_3$ and strongly reduced by blocking of GPVI (Fig. 4B). The inhibitory effect of GPVI blockage in platelet cell culture was dose-dependent (fig. S3A). The quantification of remaining soluble Aβ40 in the supernatants of platelet cell culture by Western blot analysis showed significantly increased amounts of Aβ40 when GPVI was blocked compared to untreated platelets consistent with reduced Aβ aggregate formation (Fig. 4C and D). To confirm these results, we used platelets from GPVI-deficient mice for cell culture experiments. Cultures of platelets from GPVI knockout mice displayed markedly reduced Aβ aggregate formation (Fig. 4E). In the supernatants of GPVI-deficient platelets we measured significantly increased amounts of soluble Aβ40 compared to WT platelets (Fig. 4F and G). The additional blocking of integrin $\alpha_{IIb}\beta_3$ by antibody Leo.H4 led to increasing amounts of soluble Aβ40 in the supernatant compared to GPVI deficiency alone and to complete inhibition of Aβ aggregates in cell culture (Fig. 4E to G).

Direct binding of Aβ40 to GPVI

To elucidate the mechanisms by which Aβ40 peptides induce GPVI activation, we investigated the interaction between GPVI and Aβ40. First, we used the microarray AVEXIS screening assay (25). No binding with a control protein (CD200R-BLH) but direct binding of pentameric GPVI to Aβ40 peptides was observed (Fig. 5A and B). In a second approach, we confirmed the interaction of both proteins by the use of immobilized magnetic beads coated with recombinant GPVI and incubated with soluble Aβ40. After pulldown, the association was visualized by Western blotting using antibodies to GPVI and Aβ (Fig. 4C). When Aβ40 was passed through GPVI-bound beads, a large amount of Aβ was detected along with GPVI. To verify the interaction between GPVI on platelets and Aβ40 in vitro, we incubated murine platelets with Aβ40 peptides and immunoprecipitated GPVI with the antibody JAQ1. Western blot analysis demonstrated that Aβ peptides were co-immunoprecipitated with GPVI (Fig. 4D). To show the

relevance of GPVI for Aβ binding to platelets, platelets from GPVI-deficient and WT mice were incubated with Aβ40 peptides. Using flow cytometry, binding of Aβ40 to platelets was detected by FITC labeled Aβ antibody (Fig. 4E). Binding of Aβ to GPVI deficient platelets was significantly reduced compared to WT platelets. Additionally, binding of Aβ to platelets was increased upon stimulation with both CRP and soluble Aβ40 and significantly reduced by integrin $\alpha_{IIb}\beta_3$ blocking in WT platelets. This might be due to an increased number of integrins at the platelet surface after CRP stimulation that allows augmented Aβ40 binding to integrin $\alpha_{IIb}\beta_3$.

Release of fibrinogen through Aβ-induced GPVI activation and colocalization of fibrinogen with Aβ aggregates

The most abundant of platelet secretory granules are α-granules, which contain about 300 proteins, including von Willebrand factor (vWF), integrin $\alpha_{IIb}\beta_3$ and fibrinogen (26). The release of the α-granule content is important for all platelet functions, including hemostasis, inflammation and angiogenesis (27). In a previously reported study, we showed that monomeric and oligomeric Aβ40 bound to fibrinogen and concluded that fibrinogen bridges Aβ/integrin $\alpha_{IIb}\beta_3$ complexes of platelets and contributes to the occlusion of cerebral vessels in APP23 mice, an Alzheimer's disease model (19). Thus, we analyzed the release of fibrinogen from platelet α-granules upon Aβ40 stimulation. The release of fibrinogen from platelets in response to Aβ40 was increased and inhibited by losartan comparable to blocking of integrin $\alpha_{IIb}\beta_3$ (Fig. 6A). In the presence of GPVI-blocking (JAQ1) or $\alpha_{IIb}\beta_3$ integrin-blocking (Leo.H4) antibodies, the release of fibrinogen was strongly reduced in response to Aβ40 (Fig. 6B). To characterize the impact of released fibrinogen on the formation of Aβ aggregates, we incubated murine platelets with Aβ40 for 3 days and analyzed fibrinogen and Aβ localization by immunofluorescence staining, which revealed that fibrinogen and Aβ aggregates colocalized (Fig. 6C). We also observed colocalization of Aβ aggregates and fibrinogen in cultures of human platelets (fig. S4A). Blocking of active factor X with the selective inhibitor Arixtra did not alter binding of fibrinogen or amyloid fibril aggregate formation suggesting that the conversion of fibrinogen to fibrin did not play a role in platelet mediated amyloid fibril aggregate formation (fig. S5). The inhibition of GPVI on platelets not only led to reduced aggregation of Aβ but also to less accumulation of fibrinogen in cell culture (Fig. 6C, middle panel). Additionally, the inhibition of integrin $\alpha_{IIb}\beta_3$ by blocking antibodies prevented the formation of Aβ aggregates as well as the accumulation of fibrinogen in cell culture (Fig. 6C, lower panel). To confirm the impact of GPVI on the release of fibrinogen upon Aβ40 stimulation of platelets, we used platelets from

WT and GPVI-deficient mice. Western blot analysis revealed that GPVI deficient platelets did not release fibrinogen neither upon stimulation with Aβ40 nor in response to the GPVI agonist CRP (Fig. 6D). Reduced formation of Aβ aggregates was accompanied by reduced fibrinogen in cell culture using GPVI deficient platelets compared to WT controls (Fig. 6E). Together, these results suggested that Aβ40 induced the release of fibrinogen from platelets via GPVI and the released fibrinogen co-localized with Aβ aggregates in cell culture.

Reduced Aβ-induced platelet adhesion in vivo by blocking or genetically deleting GPVI

Platelet adhesion to vascular Aβ plaques in cerebral vessels of transgenic Alzheimer's disease-model mice and enhanced Aβ40 triggered platelet adhesion at the injured vessels of wild-type mice in vivo were shown in a previous study (18). To explore the inhibitory effects of losartan on Aβ40 enhanced platelet adhesion at the vessel in vivo, we analyzed platelet adhesion at the injured carotid artery by in vivo fluorescence microscopy. Platelets from donor mice were stained with CellTracker™ Red and activated with Aβ40 in the absence or presence of losartan (Fig. 7A and Movies S1 and S2). As expected, stimulation of donor platelets with Aβ40-induced tethering and stable adhesion of platelets at sites of injury in recipient mice (Fig. 7, A to C). In contrast, treatment of donor platelets with Aβ and losartan led to a statistically significant reduction of tethered (Fig. 7B) and stable adherent (Fig. 7C) platelets at the injured vessel in recipient mice.

To confirm an important role of GPVI on Aβ40 triggered platelet adhesion at the injured vessel in vivo, we used platelets from donor mice lacking GPVI. In vivo fluorescence imaging of platelet adhesion at sites of injury in WT recipient mice showed reduced adhesion of Aβ40-stimulated GPVI-deficient platelets compared to WT controls (Fig. 7, A to C, and Movie S3).

DISCUSSION

GPVI is one of the key receptors involved in hemostasis and the prothrombotic state of acute coronary syndrome, thus targeting GPVI may be therapeutic for thrombosis. Recombinant GPVI-Fc improves left ventricular function after experimental myocardial infarction in mice (28). Injection of GPVI specific antibodies into mice leads to the depletion of the receptor and provides strong protection against arterial thrombosis (29, 30). GPVI is also implicated in vascular integrity during development and inflammation (31). Here, our study using platelets from patients and mice, revealed that GPVI may also contribute to AD through direct interaction with Aβ40 and the consequent release of fibrinogen that amplifies platelet-mediated

formation of amyloid fibrils. GPVI-blocking antibodies reduced platelet-associated amyloid aggregate formation. Aβ40 induced tyrosine phosphorylation in a GPVI-dependent, manner including the phosphorylation of LAT. Platelet aggregation, ATP release induced by Aβ40 and LAT phosphorylation were reduced in GPVI-deficient murine and human platelets. GPVI induced release of fibrinogen accounted for amyloid aggregate formation in vitro. In vivo, enhanced platelet accumulation at injured vessels after stimulation of platelets with Aβ40 was markedly reduced when we injected GPVI deficient platelets or treated platelets with losartan, a small molecule which has been described to inhibit collagen-induced platelet aggregation in mice (23, 24), inhibited Aβ40-induced platelet aggregation and ATP and fibrinogen release but had no effect on platelet-mediated amyloid aggregate formation. These results are in line with studies showing that the use of angiotensin receptor blockers, such as losartan, restore cerebrovascular dysfunction but have no effects on memory decline or AD pathology (as in, specifically, amyloidosis) (32, 33). The selective blocking of the angiotensin IV and its receptor (AngIV/AT4R)-mediated cascade is suggested to represent the underlying mechanism in losartan's benefits. However, our data suggest that the beneficial effect on cerebrovascular function is not restricted to the AngIV/AT4R cascade but rather also includes reduction of GPVI-induced platelet activation and aggregation, demonstrating broader implications of losartan. These results are in line with a study by Elaskalani and colleagues who showed reduced platelet aggregation and PLCγ2 phosphorylation in response to Aβ42 when they block GPVI by losartan (34).

Besides collagen, several GPVI ligands have been identified; these include diesel exhaust particles (DEP) and large polysaccharides, such as fucoidan and dextran sulfate (35), as well as fibrin (36). Here we provide evidence for Aβ40 binding to GPVI and acting as a regulator of GPVI signaling including tyrosine phosphorylation, ATP and fibrinogen release and platelet aggregation. Activation of GPVI was induced by direct binding of Aβ40 to the receptor and most likely not as secondary effect of, say, fibrinogen release, conversion of fibrinogen to fibrin, and fibrin-mediated GPVI activation.

To date, there is only one study that has investigated GPVI in AD. Those authors showed that, compared to healthy controls, AD patients have decreased plasma levels of soluble GPVI (sGPVI) (37). This finding is of notable interest in terms of an antithrombotic strategy, given that sGPVI could bind collagen exposed upon vessel injury and thus reduces its binding to platelet GPVI. Reduced sGPVI plasma levels imply increased GPVI exposure at the surface of AD platelets, suggesting an increased number of Aβ40-sensitive receptors at the platelet surface and thus potentially enhanced Aβ40 binding to platelets in AD patients.

Our data suggest that the binding of Aβ40 to GPVI induces the release of fibrinogen that is then incorporated into amyloid aggregates (Fig. 6). The formation of fibrin might not play a role since treatment of platelets with factor X inhibitor Arixtra did not alter platelet induced amyloid aggregate formation in culture. Studies have shown that fibrinogen only binds to human but not mouse GPVI (38, 39). Because we did not observe differences in the integration of fibrinogen into Aβ fibrils using either human or mouse platelets we do not believe that fibrinogen binding to GPVI plays a role in platelet induced amyloid aggregate formation. Fibrinogen has been identified as possible contributor to the pathology of AD, and reducing fibrinogen decreases neurovascular damage, blood-brain barrier permeability and neuroinflammation in AD (40). Fibrinogen is a cerebrovascular risk factor that is able to bind to Aβ thereby altering fibrin clot structure and degradation (41, 42). The interaction of Aβ and fibrinogen induces fibrinogen oligomerization (42). Targeting the interaction of Aβ and fibrinogen is a promising new therapeutic approach in AD (43). However, the authors had not taken into consideration that platelets might play a role by binding to fibrinogen and / or Aβ. Here, we provide evidence for platelets playing an important role in Aβ40-induced release of fibrinogen via GPVI and integrin $\alpha_{IIb}\beta_3$ and for fibrinogen being involved in platelet induced amyloid aggregate formation.

We propose that engagement of GPVI and integrin $\alpha_{IIb}\beta_3$ by Aβ40 at the platelet surface induces the formation of an Aβ fibril network that included binding of Aβ40 to GPVI and integrin $\alpha_{IIb}\beta_3$ as well as fibrinogen binding to Aβ40 and integrin $\alpha_{IIb}\beta_3$ (Fig. 7D). These different binding possibilities might induce the formation of a specific type of "clustering" of GPVI and integrin $\alpha_{IIb}\beta_3$. Therefore, it is feasible that the failure of losartan to prevent platelet-mediated Aβ aggregate formation is due to its inability to block GPVI clustering as already shown in the presence of collagen (24). However, because we previously did not observe integrin activation in the presence of Aβ40 alone (19), binding of Aβ40 to GPVI and integrin $\alpha_{IIb}\beta_3$ probably did not induce integrin inside-out signaling. According to our data, both previously published (19) and extended here, Aβ40 binds to non-activated integrin $\alpha_{IIb}\beta_3$ on the surface of platelets, and this binding is enhanced in the presence of ADP and CRP, probably because of activation-induced up-regulation of $\alpha_{IIb}\beta_3$ at the platelet surface.

Together, our findings reveal that GPVI mediates platelet-induced amyloid aggregate formation through the release of ATP and fibrinogen in response to direct binding of Aβ40 at the platelet surface. Further analysis is needed to validate whether blocking GPVI is beneficial to reduce amyloid plaque formation in cerebral vessels (as in CAA) and in brain parenchyma.

MATERIALS AND METHODS

Chemicals and antibodies

Platelets were activated with CRP (Richard Farndale, University of Cambridge, United Kingdom) or soluble Aβ40 (1-40; Bachem Peptide, cat no 4014442.1000) sequence single-letter code (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV). Aβ1-40 stock solutions with a concentration of 1 mg/ml, were solved in sterile H_2O and stored at -20 ˚C. Apyrase (grade II, from potato) and prostacyclin from Calbiochem were used for isolation. Antibodies against phosphotyrosine (Millipore clone 4G10; cat no 05-321), phospho-LAT (Tyr^{200}; Abcam cat no ab68139); Aβ1-16 (Biolegend, 6E10, cat no SIG-39320) and fibrinogen (Dako cat no A0080) were used for immunoblotting. The antibodies to LAT (cat no 9166), β-actin (cat no 4967), α-tubulin (cat no 2144), and horseradish peroxidase (HRP)-linked secondary antibodies (cat no 7074 and cat no 7076) were from Cell Signaling Technology.

Animals

Mice with targeted deletion of GPVI were provided by Jerry Ware and backcrossed to C57BL/6 mice. For the generation of homozygous WT and *Gp6*$^{-/-}$ mice, heterozygous breeding partners were mated. The animals were maintained in an environmentally controlled room at 22 ± 1 °C with a 12 h day-night cycle. Two to five mice were housed in Macrolon cages type III with *ad libitum* access to food (standard chow diet) and water. All animal experiments were conducted according the Declaration of Helsinki and approved by the Ethics Committee of the State Ministry of Agriculture, Nutrition and Forestry State of North Rhine-Westphalia, Germany (reference number: AZ 84-02.05.40.16.073; AZ 81-02.4.2019.A232).

Human platelet preparation

Platelets were prepared as previously described (19). Fresh ACD-anticoagulated blood was obtained from healthy volunteers (age of 18-50 years, from the blood bank, not AD or GPVI-deficient) and GPVI-deficient patients as indicated. Participants provided their written informed consent to participate in this study according to the Ethics Committee and the Declaration of Helsinki (study number 2018-140-KFogU). Collected blood was centrifuged at 200 x g for 10 min at room temperature. The supernatant (platelet-rich plasma; PRP) was added to phosphate buffered saline (PBS; pH 6.5, apyrase: 2.5 U/ml and 1 µM PGI_2 in 1:1 volumetric ratio and centrifuged at 1000 x g for 6 min. Platelets were resuspended in Tyrode´s-buffer solution (140 mM NaCl; 2.8 mM KCl; 12 mM $NaHCO_3$; 0.5 mM Na_2HPO_4; 5.5 mM glucose pH 7.4).

Murine platelet preparation

Murine blood was acquired by retro bulbar puncture and centrifuged at 250 x g for 5 min. The samples were centrifuged at 50 x g for 6 min to obtain PRP. PRP was washed two times (650 x g for 5 min at room temperutre), before the pellet was resuspended in Tyrode's buffer [136 mM NaCl, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.1% glucose, 0.35% bovine serum albumin (pH 7.4)] supplemented with prostacyclin (0.5 µM) and apyrase (0.02 U/ml). Before use, platelets were resuspended in the same Tyrode´s buffer supplemented with 1 mM $CaCl_2$.

Human and murine cell culture

Isolated human or murine platelets were preincubated for 15 min with 6 µg per $2*10^6$ platelets anti-mouse integrin $\alpha_{IIb}\beta_3$ antibody (Leo.H4/Rat IgG2b, emfret ANALYTICS, cat no M021-0] or 6 µg per $2*10^6$ platelets anti-mouse GPVI antibody (JAQ1 Rat IgG2a, emfret ANALYTICS, cat no M011-0) or 100 µM losartan (Tocris, cat no 3798). The final concentration of 2×10^6 platelets/well were added to 150 µl of DMEM medium (Dulbecco's modified Eagle's medium). Platelets were stimulated with 5 µM Aβ40 or 5 µg/ml CRP for 3 days at 37 °C and 5% CO_2. After three days of incubation unbound platelets were removed by rinsing with PBS. Adherent platelets were fixed with 2 % paraformaldehyde and stained against fibrillary Aβ aggregates with Congo red according to the manufacturer's protocol (Millipore cat no 101641). For Immunofluorescence staining, slides were washed with 100 µl PBS before fixed with 2% paraformaldehyde and blocked for 1 h with 5 % goat-serum in PBS. Afterwards slides were incubated overnight at 4°C with the primary antibodies against Aβ (mouse-anti-human; 6E10), fibrin-[ogen] (rabbit-anti-mouse; DAKO) and the IgG controls in a 1:100 dilution containing 1% BSA and 5% goat serum in PBS. The next day the chamber slide was washed three times with PBS and afterward incubated for 1 h at room temperature with the secondary antibodies Alexa Fluor 488- and 555-labelled (Lifetechnologies cat no A32727 and cat no A 32790) in a 1:250 dilution containing 1% BSA and 5% goat serum in PBS.

For immunoblotting analysis, the cell culture supernatants were removed and centrifuged at 10.000 x g for 10 min at 4°C. The supernatant was collected, prepared with reducing sample buffer (Laemmli buffer) and denatured at 95 °C for 5 min.

Cell lysis and immunoblotting

Platelets (60×10^6) were stimulated with 20 µM soluble Aβ40, or 5 µg/ml CRP in Tyrode's buffer (pH 7.4) at 37 °C for the indicated time. Pretreatment, when indicated, with anti-mouse integrin $\alpha_{IIb}\beta_3$ antibody (Leo.H4; emfret ANALYTICS), anti-mouse GPVI antibody (JAQ1; emfret ANALYTICS), Abciximab (Janssen-Cilag GmbH) or losartan (Cayman chemical

company) occurred at 37 °C for 15-30 min. For separation into supernatant and pellet, platelets were centrifuged at 650 x g. For platelets lysis human platelets were incubated for 15 min on ice with lysis buffer containing: 145 mM NaCl, 20 mM tris-HCl, 5 mM EDTA, 0.5 % sodium deoxycholat, 1% Triton X-100, and complete protease inhibitor (PI) cocktail (Roche cat no 5892970001). Murine platelets were incubated for 15 min on ice with lysis buffer containing 15 mM tris-HCl, 155 mM NaCl, 1 mM EDTA (pH 8.05), 0.005% NaN3, 1% IGPAL and PI. Platelet lysates (30 µl) and supernatants (30 µl) were subjected to SDS–polyacrylamide gel under reducing conditions and transferred onto nitrocellulose blotting membrane (GE Healthcare Life Sciences). Membrane was blocked using 5 % BSA or 5 % non-fat dry milk in TBST (Tris-buffered saline with 0.1 % Tween 20) and probed with the appropriate primary antibody (dilution 1:1000 in 5% BSA in TBST) and secondary (dilution 1:2500 in 5% non-fat dry milk in TBST) HRP-conjugated antibody. Band intensities were quantified in relation to untreated platelets using the FUSION-FX7 software (Vilber).

p-LAT: Under non-aggregating conditions (Apyrase 0.5U/ml, Lotrafiban 10µM and indomethacin 10µM), human and mouse platelets (1.5×10^6) were stimulated with 30 µM soluble Aβ40, 5 µg/ml CRP or 1 µg/ml Collagen in Tyrode's buffer (pH 7.4) for the indicated time at 37 °C. Cells were immediately lysed on ice with NP-40 lysis buffer (300 mM NaCl, 20mM Tris, 2mM EGTA, 2mM EDTA and 2% NP-40 detergent) in addition to the protease and phosphatase inhibitors (5mM Sodium Orthovanadate, 1mM AEBSF, 10µg/ml leupeptin, 10µg/ml aprotinin and 1µg/ml pepstatin). Platelet lysates were loaded in a gradient gel (NuPAGE 4-12%, Invitrogen) under reducing conditions and transferred onto PVDF blotting membrane (TransBlot Turbo, Bio-Rad). Membrane was blocked using 5 % BSA in TBST (TBS with 0.1 % Tween 20) and probed with the appropriate primary antibody pLAT (abcam, dilution 1:500) or α-tubulin (SIGMA, dilution 1:1000), and secondary HRP-conjugated antibody anti-mouse IgG (GE Healthcare, dilution 1:5000) or anti-rabbit IgG (GE Healthcare, dilution 1:5000). Band signals were detected using Odyssey Fc imaging system (LI-COR).

Immunoprecipitation

$1x10^9$ platelets were stimulated with 20 µM Aβ40 for 30 min at 37°C while shaken. Platelets without stimulation with Aβ40 were used as a control (resting). Murine resting and 20 µM Aβ40-stimulated platelets were lysed with 5 x lysis buffer (as described in Cell lysis and immunoblotting**)** for 10 min on ice. Afterward, the lysate was centrifuged at 10.000 x g for 10 min at 4°C to clear the lysate from remaining cell fragments. The cleared lysate was transferred to a new reaction tube and incubated with GPVI antibody or corresponding IgG control (JAQ1; emfret ANALYTICS; Mouse IgG_{2b} Cell Signaling) for 1 h at 4°C. Samples

were transferred to a new reaction tube and incubated with washed G-Sepharose protein overnight at 4 °C. Samples were washed three times: first time with immunoprecipitation buffer (15 mM Tris-HCl; 155 mM NaCl, 1 mM EDTA and 0,005 % NaN3) containing additionally 1% IGPAL, second and third time only with immunoprecipitation buffer before adding 2x Laemmli, containing 5% mercaptoethanol and incubated at 95°C for 5 min. After centrifugation at 10.000 x g for 2 min supernatants were removed and analyzed via immunoblotting against Aβ (Biolegend, 6E10) and GPVI (R&D Systems cat no AF6758).

Pull-down

Recombinant GPVI (R&D Systems cat no 6758-GP-050) was covalently immobilized to Pierce NHS-Activated Magnetic Beads according to the manufacturer's information (Thermo Scientific cat no 88802). Protein solution with and without 20 µM Aβ40 was added to the GPVI coupled beads and incubated at room temperature on a rotator for 1-2 hours. Beads were collected with the magnetic stand and washed for three times with wash buffer (TBS with 0.05% Tween 20 Detergent) and afterward washed with ultrapure water. For protein elution beads were washed with 100 µl elution buffer (0.1M glycine, pH 2.0) and pH was neutralized by adding 10 µl neutralization buffer (1M Tris; pH 9). Laemmli buffer was added and samples were analyzed via immunoblotting under reducing conditions against Aβ (Biolegend, 6E10) and GPVI (R&D Systems cat no AF6758).

AVEXIS screening

Aβ40 peptides or CD200R bait proteins were incubated in MaxiSorp 96-well microtiter plates (Nunc) for 1 h, then blocked with 1% BSA for 30 min. The peptide-coated plate was incubated with full length recombinant soluble pentameric (s5) GPVI and s5CD200 for 1 h. Three wash steps were performed between each incubation using PBS with 0.1% Tween 20. After addition of 125 µg/ml nitrocefin (#N005, Toku-e) was added and incubated for 1 h, absorbance was measured at 485 nm on VERSA max microplate reader (Molecular Devices).

Platelet aggregation and ATP release

Aggregation was measured as percentage light transmission compared to Tyrode's buffer (as=100%) using Chrono-Log dual channel lumi-aggregometer (model 700) at 37 °C stirring at 1000 rpm. Human ATP-release was assessed applying a luciferin/luciferase bioluminescent assay and calculated using a provided ATP standard protocol (all Chrono-Log). Murine ATP-release was measured using ATP Bioluminescence Assay Kit HS II (Roche; cat no 11699709001) according to the manufacturer's information and normalized to resting.

Flow Cytometry

Flow Cytometry was performed as described (18, 44). Analysis of Aβ40 binding to platelets surface was carried out using fluorophore labeled antibodies for Aβ (anti- Aβ-FITC; Santa Cruz cat no sc-28365). 25 µl of washed blood samples were diluted in Tyrode´s buffer with 1 mM $CaCl_2$ and stimulated with indicated agonist (5 µM Aβ40; 5 µg/ml CRP) and antibody at room temperature for 15 min. Reaction was stopped using 300 µl PBS. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Ligation of the carotid artery model

Carotid ligation in mice were performed as described elsewhere (18). Platelets from WT and *Gp6*$^{-/-}$ donor mice were stained with CellTracker™ Red CMTPX (Invitrogen) according to the manufacturer's guidelines and incubated with losartan (100µM) or vehicle and Aβ (50µg/ml) for 30 min. WT littermates mice were anaesthetized using Ketamine (Zoetis) and Xylacine (WDT) and put on a heating pad. The right common carotid artery was prepared and after intravenous injection of fluorescently labelled and treated platelets a film of 30 s was taken using a DM6FS microscope (Leica Microsystems, Wetzlar, Germany). Afterward the carotid artery was ligated vigorously for 5 min, thus inducing vascular injury. The interaction of the fluorescent platelets with the injured vessel wall was visualized 20 min after ligation by in vivo video microscopy. Tethering and adherent cells were counted as means from 10 different pictures throughout the film with the same time span between these pictures, but always in the same phase of vessel pulsation.

Statistical analysis

Data are provided as arithmetic means ± SEM. Significant differences were calculated using the two-way ANOVA with Bonferroni's multiple comparison post hoc test; one-way ANOVA with Dunnett's post hoc test or students t-test as indicated in the figure legends. Outliers were excluded using Grubb´s test.

SUPPLEMENTARY MATERIALS

Fig. S1. Time-dependent LAT phosphorylation of human platelets stimulated with Aβ40.

Fig. S2. Different concentrations of losartan on platelet cell cultures.

Fig. S3. Reduced amyloid aggregate formation through GPVI inhibition in a concentration-dependent manner.

Fig. S4. Immunofluorescence staining of fibrinogen and Aβ in human and murine platelet cell cultures.

Fig. S5. No alteration of amyloid formation upon inhibition of active factor Xa.

Movie S1. Video of in vivo WT platelet adhesion at the injured carotid artery.

Movie S2. Video of in vivo WT platelet adhesion at the injured carotid artery after losartan treatment.

Movie S3. Video of in vivo *Gp6*$^{-/-}$ platelet adhesion at the injured carotid artery.

REFERENCES AND NOTES

1. M. Prince, G. C. Ali, M. Guerchet, A. M. Prina, E. Albanese, and Y. T. Wu, Recent global trends in the prevalence and incidence of dementia, and survival with dementia. *Alzheimers Res Ther* 8: 23 (2016).
2. G. Rakesh, S. T. Szabo, G. S. Alexopoulos, and A. S. Zannas, Strategies for dementia prevention: latest evidence and implications. *Ther Adv Chronic Dis* 8: 121-136 (2017).
3. A. M. Clarfield, The decreasing prevalence of reversible dementias: an updated meta-analysis. *Arch Intern Med* 163: 2219-2229 (2003).
4. D. J. Selkoe, and J. Hardy, The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med* 8: 595-608 (2016).
5. D. J. Selkoe, Alzheimer's disease. *Cold Spring Harb Perspect Biol* 3 (2011).
6. H. Braak, E. Braak, D. Yilmazer, R. A. de Vos, E. N. Jansen, and J. Bohl, Pattern of brain destruction in Parkinson's and Alzheimer's diseases. *J Neural Transm (Vienna)* 103: 455-490 (1996).
7. A. Serrano-Pozo, M. P. Frosch, E. Masliah, and B. T. Hyman, Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* 1: a006189 (2011).
8. M. Sochocka, E. S. Koutsouraki, K. Gasiorowski, and J. Leszek, Vascular oxidative stress and mitochondrial failure in the pathobiology of Alzheimer's disease: a new approach to therapy. *CNS Neurol Disord Drug Targets* 12: 870-881 (2013).
9. M. Z. Wojtukiewicz, E. Sierko, D. Hempel, S. C. Tucker, and K. V. Honn, Platelets and cancer angiogenesis nexus. *Cancer Metastasis Rev* 36: 249-262 (2017).
10. T. G. Walsh, P. Metharom, and M. C. Berndt, The functional role of platelets in the regulation of angiogenesis. *Platelets* 26: 199-211 (2015).
11. M. R. Thomas, and R. F. Storey, The role of platelets in inflammation. *Thromb Haemost* 114: 449-458 (2015).
12. C. Goubau, G. M. Buyse, C. Van Geet, and K. Freson, The contribution of platelet studies to the understanding of disease mechanisms in complex and monogenetic neurological disorders. *Dev Med Child Neurol* 56: 724-731 (2014).
13. E. Asor, and D. Ben-Shachar, Platelets: A possible glance into brain biological processes in schizophrenia. *World J Psychiatry* 2: 124-133 (2012).
14. M. Padmakumar, E. Van Raes, C. Van Geet, and K. Freson, Blood platelet research in autism spectrum disorders: In search of biomarkers. *Res Pract Thromb Haemost* 3: 566-577 (2019).
15. M. Behari, and M. Shrivastava, Role of platelets in neurodegenerative diseases: a universal pathophysiology. *Int J Neurosci* 123: 287-299 (2013).
16. K. Stellos, V. Panagiota, A. Kogel, T. Leyhe, M. Gawaz, and C. Laske, Predictive value of platelet activation for the rate of cognitive decline in Alzheimer's disease patients. *J Cereb Blood Flow Metab* 30: 1817-1820 (2010).
17. A. Jarre, N. S. Gowert, L. Donner, P. Munzer, M. Klier, O. Borst, M. Schaller, F. Lang, C. Korth, and M. Elvers, Pre-activated blood platelets and a pro-thrombotic

phenotype in APP23 mice modeling Alzheimer's disease. *Cell Signal* 26: 2040-2050 (2014).
18. N. S. Gowert, L. Donner, M. Chatterjee, Y. S. Eisele, S. T. Towhid, P. Munzer, B. Walker, I. Ogorek, O. Borst, M. Grandoch, M. Schaller, J. W. Fischer, M. Gawaz, S. Weggen, F. Lang, M. Jucker, and M. Elvers, Blood platelets in the progression of Alzheimer's disease. *PLoS One* 9: e90523 (2014).
19. L. Donner, K. Falker, L. Gremer, S. Klinker, G. Pagani, L. U. Ljungberg, K. Lothmann, F. Rizzi, M. Schaller, H. Gohlke, D. Willbold, M. Grenegard, and M. Elvers, Platelets contribute to amyloid-beta aggregation in cerebral vessels through integrin alphaIIbbeta3-induced outside-in signaling and clusterin release. *Sci Signal* 9: ra52 (2016).
20. L. Donner, L. Gremer, T. Ziehm, C. G. W. Gertzen, H. Gohlke, D. Willbold, and M. Elvers, Relevance of N-terminal residues for amyloid-beta binding to platelet integrin alphaIIbbeta3, integrin outside-in signaling and amyloid-beta fibril formation. *Cell Signal* 50: 121-130 (2018).
21. J. M. Pasquet, B. Gross, L. Quek, N. Asazuma, W. Zhang, C. L. Sommers, E. Schweighoffer, V. Tybulewicz, B. Judd, J. R. Lee, G. Koretzky, P. E. Love, L. E. Samelson, and S. P. Watson, LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. *Mol Cell Biol* 19: 8326-8334 (1999).
22. C. Schulz, N. V. Leuschen, T. Frohlich, M. Lorenz, S. Pfeiler, C. A. Gleissner, E. Kremmer, M. Kessler, A. G. Khandoga, B. Engelmann, K. Ley, S. Massberg, and G. J. Arnold, Identification of novel downstream targets of platelet glycoprotein VI activation by differential proteome analysis: implications for thrombus formation. *Blood* 115: 4102-4110 (2010).
23. P. Jiang, S. Loyau, M. Tchitchinadze, J. Ropers, G. Jondeau, and M. Jandrot-Perrus, Inhibition of Glycoprotein VI Clustering by Collagen as a Mechanism of Inhibiting Collagen-Induced Platelet Responses: The Example of Losartan. *PLoS One* 10: e0128744 (2015).
24. M. B. Onselaer, M. Nagy, C. Pallini, J. A. Pike, G. Perrella, L. G. Quintanilla, J. A. Eble, N. S. Poulter, J. W. M. Heemskerk, and S. P. Watson, Comparison of the GPVI inhibitors losartan and honokiol. *Platelets*: 1-11 (2019).
25. Y. Sun, M. Gallagher-Jones, C. Barker, and G. J. Wright, A benchmarked protein microarray-based platform for the identification of novel low-affinity extracellular protein interactions. *Anal Biochem* 424: 45-53 (2012).
26. Y. Hou, N. Carrim, Y. Wang, R. C. Gallant, A. Marshall, and H. Ni, Platelets in hemostasis and thrombosis: Novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis. *J Biomed Res* 29 (2015).
27. P. Blair, and R. Flaumenhaft, Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev* 23: 177-189 (2009).
28. T. Schonberger, M. Ziegler, O. Borst, I. Konrad, B. Nieswandt, S. Massberg, C. Ochmann, T. Jurgens, P. Seizer, H. Langer, G. Munch, M. Ungerer, K. T. Preissner, M. Elvers, and M. Gawaz, The dimeric platelet collagen receptor GPVI-Fc reduces platelet adhesion to activated endothelium and preserves myocardial function after transient ischemia in mice. *Am J Physiol Cell Physiol* 303: C757-766 (2012).
29. B. Nieswandt, V. Schulte, W. Bergmeier, R. Mokhtari-Nejad, K. Rackebrandt, J. P. Cazenave, P. Ohlmann, C. Gachet, and H. Zirngibl, Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med* 193: 459-469 (2001).

30. S. Gruner, M. Prostredna, M. Koch, Y. Miura, V. Schulte, S. M. Jung, M. Moroi, and B. Nieswandt, Relative antithrombotic effect of soluble GPVI dimer compared with anti-GPVI antibodies in mice. *Blood* 105: 1492-1499 (2005).
31. R. H. Lee, and W. Bergmeier, Platelet immunoreceptor tyrosine-based activation motif (ITAM) and hemITAM signaling and vascular integrity in inflammation and development. *J Thromb Haemost* 14: 645-654 (2016).
32. B. Ongali, N. Nicolakakis, X. K. Tong, T. Aboulkassim, H. Imboden, and E. Hamel, Enalapril Alone or Co-Administered with Losartan Rescues Cerebrovascular Dysfunction, but not Mnemonic Deficits or Amyloidosis in a Mouse Model of Alzheimer's Disease. *J Alzheimers Dis* 51: 1183-1195 (2016).
33. J. Royea, L. Zhang, X. K. Tong, and E. Hamel, Angiotensin IV Receptors Mediate the Cognitive and Cerebrovascular Benefits of Losartan in a Mouse Model of Alzheimer's Disease. *J Neurosci* 37: 5562-5573 (2017).
34. O. Elaskalani, I. Khan, M. Morici, C. Matthysen, M. Sabale, R. N. Martins, G. Verdile, and P. Metharom, Oligomeric and fibrillar amyloid beta 42 induce platelet aggregation partially through GPVI. *Platelets* 29: 415-420 (2018).
35. O. M. Alshehri, S. Montague, S. Watson, P. Carter, N. Sarker, B. K. Manne, J. L. Miller, A. B. Herr, A. Y. Pollitt, C. A. O'Callaghan, S. Kunapuli, M. Arman, C. E. Hughes, and S. P. Watson, Activation of glycoprotein VI (GPVI) and C-type lectin-like receptor-2 (CLEC-2) underlies platelet activation by diesel exhaust particles and other charged/hydrophobic ligands. *Biochem J* 468: 459-473 (2015).
36. O. M. Alshehri, C. E. Hughes, S. Montague, S. K. Watson, J. Frampton, M. Bender, and S. P. Watson, Fibrin activates GPVI in human and mouse platelets. *Blood* 126: 1601-1608 (2015).
37. C. Laske, T. Leyhe, E. Stransky, G. W. Eschweiler, A. Bueltmann, H. Langer, K. Stellos, and M. Gawaz, Association of platelet-derived soluble glycoprotein VI in plasma with Alzheimer's disease. *J Psychiatr Res* 42: 746-751 (2008).
38. P. H. Mangin, M. B. Onselaer, N. Receveur, N. Le Lay, A. T. Hardy, C. Wilson, X. Sanchez, S. Loyau, A. Dupuis, A. K. Babar, J. L. Miller, H. Philippou, C. E. Hughes, A. B. Herr, R. A. Ariens, D. Mezzano, M. Jandrot-Perrus, C. Gachet, and S. P. Watson, Immobilized fibrinogen activates human platelets through glycoprotein VI. *Haematologica* 103: 898-907 (2018).
39. A. Slater, G. Perrella, M. B. Onselaer, E. M. Martin, J. S. Gauer, R. G. Xu, J. W. Heemskerk, R. A. S. Ariens, and S. P. Watson, Does fibrin(ogen) bind to monomeric or dimeric GPVI, or not at all? *Platelets* 30: 281-289 (2019).
40. M. Cortes-Canteli, and S. Strickland, Fibrinogen, a possible key player in Alzheimer's disease. *J Thromb Haemost* 7 Suppl 1: 146-150 (2009).
41. M. Cortes-Canteli, J. Paul, E. H. Norris, R. Bronstein, H. J. Ahn, D. Zamolodchikov, S. Bhuvanendran, K. M. Fenz, and S. Strickland, Fibrinogen and beta-amyloid association alters thrombosis and fibrinolysis: a possible contributing factor to Alzheimer's disease. *Neuron* 66: 695-709 (2010).
42. H. J. Ahn, D. Zamolodchikov, M. Cortes-Canteli, E. H. Norris, J. F. Glickman, and S. Strickland, Alzheimer's disease peptide beta-amyloid interacts with fibrinogen and induces its oligomerization. *Proc Natl Acad Sci U S A* 107: 21812-21817 (2010).
43. H. J. Ahn, J. F. Glickman, K. L. Poon, D. Zamolodchikov, O. C. Jno-Charles, E. H. Norris, and S. Strickland, A novel Abeta-fibrinogen interaction inhibitor rescues altered thrombosis and cognitive decline in Alzheimer's disease mice. *J Exp Med* 211: 1049-1062 (2014).
44. I. Pleines, M. Elvers, A. Strehl, M. Pozgajova, D. Varga-Szabo, F. May, A. Chrostek-Grashoff, C. Brakebusch, and B. Nieswandt, Rac1 is essential for phospholipase C-gamma2 activation in platelets. *Pflugers Arch* 457: 1173-1185 (2009).

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Figure legends

Fig. 1. Aβ40 stimulates tyrosine and LAT phosphorylation in a GPVI- and integrin $\alpha_{IIb}\beta_3$ -dependent manner. (**A**) Western blotting for tyrosine phosphorylation (antibody 4G10) in isolated human platelets at rest or upon stimulation with collagen related peptide (CRP, 5 µg/ml) or Aβ40 (20 µM) for 120 sec. In lane 4, as indicated, cultures were pretreated with the integrin $\alpha_{IIb}\beta_3$ antibody abciximab (0.5 µg per 1 Mio cells) for 15 min at room temperature. β-Actin served as loading control; n=5 donors. (**B**) Western blotting for LAT phosphorylation in human platelets treated as described in (A) for 30 or 120 sec. Total LAT served as loading control; n=5 donors. (**C**) Western blotting for LAT phosphorylation in platelets isolated from a control donor and a GPVI-deficient patient; cells were unperturbed (resting) or stimulated with collagen (1 µg/ml) or Aβ40 (30 µM). α-Tubulin served as loading control; n=2 GPVI-deficient patients and n=2 healthy controls. (**D**) Western blotting for LAT phosphorylation in isolated *Gp6*$^{-/-}$ and WT platelets stimulated with CRP (5 µg/ml) or Aβ40 (20 µM) for 30 or 120 sec. αTubulin served as loading control; n=6-7 mice per group.

Fig. 2. Reduced ATP release of GPVI-deficient platelets in response to Aβ40. (**A and B**) Representative ATP release curves **(A)** and analysis **(B)** in human platelets pretreated (gray traces) with losartan (100 µM) for 20 min then stimulated with CRP (5 µg/ml) or Aβ40 (20 µM). Data are mean ± SEM from n=5 donors; two-way ANOVA with Bonferroni's multiple comparison post hoc test: **p ≤0.01; ***p ≤0.001. (**C**) ATP release in WT and *Gp6*$^{-/-}$ platelets

at rest, treated with CRP (5 µg/ml), Aβ40 (10 µM), or Aβ40 pretreated with integrin $\alpha_{IIb}\beta_3$-blocking antibody Leo.H4 for 20 min. Data are mean ± SEM from 6 to 7 mice per group; CRP stimulated platelets served as controls; two-way ANOVA with Bonferroni´s multiple comparison post hoc test: *p< 0.05; **p<0.01.

Fig. 3. Reduced aggregation of GPVI-deficient platelets in response to Aβ40. (**A and B**) Representative aggregation curves (A) and the quantified maximum aggregation of platelets treated with CRP (5µg/ml) or Aβ40 (20 µM) in the presence or absence of losartan (100 µM pretreatment for 20 min). Data are mean ± SEM from n=3-4 donors; two-way ANOVA with Bonferroni's multiple comparison post hoc test, ***p<0.001. (**C and D**) As described in (A and B) in control-donor and GPVI-deficient patient platelets stimulated with Aβ40 (30 µM). Data are mean and range from 2 GPVI deficient patients and 2 healthy controls. (**E**) As described in (A and B) in platelets isolated from WT and *Gp6*$^{-/-}$ mice and treated with CRP (5 µg/ml) or Aβ40 (10 µM). Data are mean ± SEM from n=5 mice per group; two-way ANOVA with Bonferroni's multiple comparison post hoc test: ***p<0.001.

Fig. 4. Inhibition or genetic deletion of GPVI decreases amyloid aggregate formation. (**A**) Isolated human platelets were incubated with Aβ40 (5 µM) at 37°C for 3 days. Afterward amyloid aggregates were stained by congo red. Representative pictures of congo red stained amyloid aggregates platelet culture in the presence or absence of losartan (100 µM). Scale bar, 50 µm, n=5experiments. (**B**) Samples of murine platelets treated as in (A) were stained with congo red to visualize amyloid aggregates in the presence of either GPVI-blocking antibody JAQ1 or integrin $\alpha_{IIb}\beta_3$-blocking antibody Leo.H4 (each at 6 µg per 2 per $2*10^6$ cells). Scale bar, 50 µm, n=5 experiments. (**C and D**) Corresponding Western blotting and quantification of soluble Aβ in supernatants from murine platelets cultured as in (B). Leo.H4-treated platelets served as control. Data are mean ± SEM from n=5 experiments; Students t-test, *p ≤ 0.05. (**E**) Congo red staining of amyloid aggregates in cultures of platelets from WT and *Gp6*$^{-/-}$ mice without Aβ40 and with Aβ40 (5 µM) in the presence or absence of Leo.H4 (6 µg per per 2 per $2*10^6$cells). Scale bar, 50 µm, n=5 miceper group. (**F and G**) Representative Western blots (F) and quantification (G) of soluble Aβ in supernatants from WT and GPVI$^{-/-}$ murine platelet cultures of remaining soluble Aβ. Controls lacking Aβ40 (lane 1 in the blot) were not regarded in the analysis. Data are mean ± SEM from n=5 mice per group two-way ANOVA with Bonferroni's post hoc test, *p< 0.05.

Fig. 5. Aβ40 binds to GPVI. (**A**) Interaction screening using AVEXIS (Avidity-based Extracellular Interaction Screen). Biotinylated bait peptides Aβ40 (CD200R-BLH is used as control) are arrayed on the surface of a streptavidin coated plate and incubated with pentameric prey protein s5-GPVI (s5-CD200 is used as control). Interaction produces a color change to red. (**B**) Corresponding quantification of the colorimetric change after prey-binding at 485 nm as represented in (A). n=5experiments , two-way ANOVA with Bonferroni's post hoc test, ***p< 0.001 (**C**) Pulldown was accomplished using immobilized GPVI magnetic beads and incubated without and withAβ40 (20 µM). Uncoated beads served as control. Immunoprecipitates were blotted against Aβ (6E10) and GPVI. Input = cell lysate. n=3 experiments. (**D**) Isolated platelets were stimulated with Aβ40 (20 µM) and immunoprecipitated with GPVI antibody. Immunoprecipitates were analyzed via Western blotting against Aβ and GPVI. N = 3. (**E**) WT and *Gp6*$^{-/-}$ platelets were preincubated with Aβ40 (5 µM), followed by an incubation with anti-Aβ-FITC antibody. When indicated platelets were pretreated with integrin $\alpha_{IIb}\beta_3$-blocking antibody Leo.H4. Binding of Aβ to platelet surface was measured by flow cytometry (n = 9-13 mice per group; mean ± SEM; one-way ANOVA with Dunnett's post hoc test within every group, *p< 0.05, **p< 0.01).

Fig. 6. Fibrinogen release in response to Aβ and colocalization with amyloid aggregates in cell culture. (**A**) Western blotting of fibrinogen release in murine platelets upon stimulation with CRP (5 µg/ml) or Aβ40 (20 µM) (pretreated as indicated with integrin $\alpha_{IIb}\beta_3$ blocking antibody Leo.H4 (0.5 µg per 1 Mio cells) or with losartan (100 µM)). β-Actin served as loading control; representative images of n=3 experiments. (**B**) Western blotting of fibrinogen release in murine platelets as described in (A) pretreated with GPVI-blocking antibody JAQ1 and with integrin $\alpha_{IIb}\beta_3$-blocking antibody Leo.H4 (each 0.5 µg per 1 Mio cells). Representative Western blot of n=3 experiments. (**C**) Murine platelets were incubated with Aβ40 (5 µM) at 37°C for 3 days. When indicated, platelets were pretreated with GPVI-blocking antibody JAQ1 or integrin $\alpha_{IIb}\beta_3$ antibody Leo.H4. Immunostaining against amyloid β aggregates (green) and fibrinogen (red) visualizes colocalization. Scale bar, 20 µm. Representative images of n=3 experiments. (**D**) Western blot analysis of fibrinogen release in platelets from WT and *Gp6*$^{-/-}$ mice upon stimulation with CRP (5 µg/ml) or Aβ40 (20 µM). When indicated, platelets were pretreated with integrin $\alpha_{IIb}\beta_3$ antibody Leo.H4. Representative images of n=3 mice per group. (**E**) Platelets from WT and *Gp6*$^{-/-}$mice were incubated with Aβ40 (5 µM) at 37°C for 3 days in the presence and absence of integrin $\alpha_{IIb}\beta_3$ blocking antibody Leo.H4. Immunofluorescence

staining of fibrinogen (red) and Aβ (green). Scale bar, 20 µm. Representative images of n=3 mice per group.

Fig. 7. Blocking or deletion of GPVI reduces Aβ-induced platelet adhesion in vivo. (**A**) Images of stable adherent and tethering Aβ40-activated platelets 20 min after carotid artery ligation in vivo. WT platelets were either incubated only with Aβ40 (top row) or were pretreated with losartan then incubated with Aβ40 (middle row), and *Gp6*$^{-/-}$ platelets were incubated with only Aβ40. Carotid artery vessel wall is outlined using dotted lines. Arrows indicate adherent platelets. N=4-5 mice per group. Scale bar, 100 µm. (**B and C**) Quantification of tethering **(B)** and stable adherent **(C)** Aβ40-activated platelets. Data are mean± SEM; n = 4-5 mice per group. One-way ANOVA with Dunnett's post hoc test; *p≤0.05, **p≤0.01. (**D**) Tentative schematic illustration. Direct binding of Aβ40 to the collagen receptor GPVI ("1") initiates phosphorylation of LAT ("2") leading to secretion of granules and thus to the release of ATP, ADP, and fibrinogen ("3"). Activation of GPVI and binding of ADP to the $P2Y_{12}$ receptor induces a shifting of integrin $\alpha_{IIb}\beta_3$ from a closed (inactive) to open (active) form leading to enhanced binding of Aβ to integrin $\alpha_{IIb}\beta_3$. Released fibrinogen bridges binding of soluble Aβ to GPVI and integrin $\alpha_{IIb}\beta_3$ to induce the formation of amyloid aggregates at the platelet surface ("4").