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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 ($A\beta$) deposition in the brain parenchyma are hallmarks of Alzheimer's disease (AD). We previously reported that platelets contribute to $A\beta$ aggregation in cerebral vessels by secreting clusterin in response to the binding of $A\beta$ 40 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 $A\beta$ 40 bound to GPVI and induced tyrosine phosphorylation on target proteins that mediated the release of ATP and fibrinogen, resulting in platelet aggregation. Binding of $A\beta$ 40 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 A β -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 A β 40-stimulated platelet aggregation, although it did not reduce the formation of amyloid aggregates. Our findings indicate that A β 40 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 ($A\beta$) 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 $A\beta$ 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 $A\beta_{40}$ into fibrillar $A\beta$ in vitro indicates a direct impact of platelets in the aggregation property of $A\beta_{40}$ peptides (18-20). Previously we have demonstrated platelets contribute to amyloid- β aggregation through binding of $A\beta_{40}$ 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 $A\beta_{40}$ in cultured cells (19). An important indication of the involvement of platelets in $A\beta$ 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 $A\beta$ 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 A β .

RESULTS

Phosphorylation of tyrosine residues in LAT and other proteins by GPVI and integrin $\alpha_{IIb}\beta_3$ in response to A β 40 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 A β 40 induced a similar pattern of tyrosine phosphorylation compared to the stimulation of platelets with CRP as shown by Western blot analysis (Fig. 1A). A β 40 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 A β 40 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 A β 40. 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 A β 40 stimulation (Fig. 1C). However, A β 40 induced phosphorylation of LAT was higher than upon activation with low concentration of collagen. Moreover, we studied the effect of A β 40 in mouse platelets deficient in GPVI. Compared to wild type platelets, GPVI-deficient platelets failed to induce phosphorylation of LAT both upon CRP and A β 40 activation (Fig. 1D). These results indicate the ability of A β 40 to activate GPVI in human and mouse platelets.

Reduced ATP release of GPVI-deficient platelets in response to A β 40

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 A β 40, we measured the release of ATP upon A β 40 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 A β 40 induced platelet stimulation. A β 40 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 A β 40 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 A β 40 (Fig. 2, A and B). WT and GPVI-knockout mice were analyzed to confirm that A β 40 induces a release of ATP release via GPVI. The release of ATP in response to CRP or A β 40 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, A β 40 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 A β 40

Next we analyzed platelet aggregation after A β 40 stimulation. A β 40 induced platelet aggregation that was comparable to that induced by CRP (Fig. 3A). Additionally, we analyzed the effect of losartan on A β 40-induced platelet aggregation (Fig. 3A and B). In agreement with reported data, losartan significantly inhibited CRP- and A β 40-induced platelet aggregation (Fig. 3A and B). To confirm the role of GPVI in A β 40-induced platelet aggregation, we used platelets from patients with GPVI deficiency. As expected, these platelets showed no platelet aggregation in response to A β 40 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 A β 40 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 A β 40 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 A β 40, suggesting that A β 40 can induce platelet aggregation without GPVI. These results demonstrated that activation of GPVI by A β 40 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 A β 40 to fibrillar A β aggregates while blocking of integrin $\alpha_{IIb}\beta_3$ on the surface of platelets prevents A β aggregate formation (19). To investigate a role of GPVI in platelet mediated A β aggregate formation, human platelets were pretreated with losartan and incubated with soluble, synthetic A β 40 for 3

days. The formation of fibrillar A β aggregates were analyzed by congo red staining. Although A β 40 induced platelet aggregation and ATP release were reduced in the presence of losartan, we did not observe alterations in A β aggregation in platelet cell culture (Fig. 4A). Neither daily addition nor different concentrations of losartan were able to reduce fibrillar A β 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 A β aggregate formation. The formation of A β 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 A β 40 in the supernatants of platelet cell culture by Western blot analysis showed significantly increased amounts of A β 40 when GPVI was blocked compared to untreated platelets consistent with reduced A β 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 A β aggregate formation (Fig. 4E). In the supernatants of GPVI-deficient platelets we measured significantly increased amounts of soluble A β 40 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 A β 40 in the supernatant compared to GPVI deficiency alone and to complete inhibition of A β aggregates in cell culture (Fig. 4E to G).

Direct binding of A β 40 to GPVI

To elucidate the mechanisms by which A β 40 peptides induce GPVI activation, we investigated the interaction between GPVI and A β 40. First, we used the microarray AVEKIS screening assay (25). No binding with a control protein (CD200R-BLH) but direct binding of pentameric GPVI to A β 40 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 A β 40. After pulldown, the association was visualized by Western blotting using antibodies to GPVI and A β (Fig. 4C). When A β 40 was passed through GPVI-bound beads, a large amount of A β was detected along with GPVI. To verify the interaction between GPVI on platelets and A β 40 in vitro, we incubated murine platelets with A β 40 peptides and immunoprecipitated GPVI with the antibody JAQ1. Western blot analysis demonstrated that A β peptides were co-immunoprecipitated with GPVI (Fig. 4D). To show the

relevance of GPVI for A β binding to platelets, platelets from GPVI-deficient and WT mice were incubated with A β 40 peptides. Using flow cytometry, binding of A β 40 to platelets was detected by FITC labeled A β antibody (Fig. 4E). Binding of A β to GPVI deficient platelets was significantly reduced compared to WT platelets. Additionally, binding of A β to platelets was increased upon stimulation with both CRP and soluble A β 40 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 A β 40 binding to integrin $\alpha_{IIb}\beta_3$.

Release of fibrinogen through A β -induced GPVI activation and colocalization of fibrinogen with A β 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 A β 40 bound to fibrinogen and concluded that fibrinogen bridges A β /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 A β 40 stimulation. The release of fibrinogen from platelets in response to A β 40 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 A β 40 (Fig. 6B). To characterize the impact of released fibrinogen on the formation of A β aggregates, we incubated murine platelets with A β 40 for 3 days and analyzed fibrinogen and A β localization by immunofluorescence staining, which revealed that fibrinogen and A β aggregates colocalized (Fig. 6C). We also observed colocalization of A β 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 A β 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 A β 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 A β 40 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 A β 40 nor in response to the GPVI agonist CRP (Fig. 6D). Reduced formation of A β aggregates was accompanied by reduced fibrinogen in cell culture using GPVI deficient platelets compared to WT controls (Fig. 6E). Together, these results suggested that A β 40 induced the release of fibrinogen from platelets via GPVI and the released fibrinogen co-localized with A β aggregates in cell culture.

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

Platelet adhesion to vascular A β plaques in cerebral vessels of transgenic Alzheimer's disease-model mice and enhanced A β 40 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 A β 40 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 A β 40 in the absence or presence of losartan (Fig. 7A and Movies S1 and S2). As expected, stimulation of donor platelets with A β 40-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 A β 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 A β 40 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 A β 40-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 A β 40 and the consequent release of fibrinogen that amplifies platelet-mediated

formation of amyloid fibrils. GPVI-blocking antibodies reduced platelet-associated amyloid aggregate formation. A β 40 induced tyrosine phosphorylation in a GPVI-dependent, manner including the phosphorylation of LAT. Platelet aggregation, ATP release induced by A β 40 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 A β 40 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 A β 40-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 PLC γ 2 phosphorylation in response to A β 42 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 A β 40 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 A β 40 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 A β 40-sensitive receptors at the platelet surface and thus potentially enhanced A β 40 binding to platelets in AD patients.

Our data suggest that the binding of A β 40 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 A β 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 A β thereby altering fibrin clot structure and degradation (41, 42). The interaction of A β and fibrinogen induces fibrinogen oligomerization (42). Targeting the interaction of A β 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 A β . Here, we provide evidence for platelets playing an important role in A β 40-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 A β 40 at the platelet surface induces the formation of an A β fibril network that included binding of A β 40 to GPVI and integrin $\alpha_{IIb}\beta_3$ as well as fibrinogen binding to A β 40 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 A β 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 A β 40 alone (19), binding of A β 40 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, A β 40 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 A β 40 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 A β 40 (1-40; Bachem Peptide, cat no 4014442.1000) sequence single-letter code (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV). A β 1-40 stock solutions with a concentration of 1 mg/ml, were solved in sterile H₂O 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 (Tyr²⁰⁰; Abcam cat no ab68139); A β 1-16 (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 μ M PGI₂ 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 NaHCO₃; 0.5 mM Na₂HPO₄; 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 temperature), before the pellet was resuspended in Tyrode's buffer [136 mM NaCl, 0.4 mM Na₂HPO₄, 2.7 mM KCl, 12 mM NaHCO₃, 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 CaCl₂.

Human and murine cell culture

Isolated human or murine platelets were preincubated for 15 min with 6 μg per 2*10⁶ platelets anti-mouse integrin α_{IIb}β₃ antibody (Leo.H4/Rat IgG2b, emfret ANALYTICS, cat no M021-0) or 6 μg per 2*10⁶ 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 × 10⁶ 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% CO₂. 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 × 10⁶) 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 α_{IIb}β₃ 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

1×10^9 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 A β (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 μ M A β 40 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 μ l elution buffer (0.1M glycine, pH 2.0) and pH was neutralized by adding 10 μ l neutralization buffer (1M Tris; pH 9). Laemmli buffer was added and samples were analyzed via immunoblotting under reducing conditions against A β (Biolegend, 6E10) and GPVI (R&D Systems cat no AF6758).

AVEXIS screening

A β 40 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 μ g/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 A β 40 binding to platelets surface was carried out using fluorophore labeled antibodies for A β (anti- A β -FITC; Santa Cruz cat no sc-28365). 25 μ l of washed blood samples were diluted in Tyrode's buffer with 1 mM CaCl₂ and stimulated with indicated agonist (5 μ M A β 40; 5 μ g/ml CRP) and antibody at room temperature for 15 min. Reaction was stopped using 300 μ l 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 (100 μ M) or vehicle and A β (50 μ g/ml) for 30 min. WT littermates mice were anaesthetized using Ketamine (Zoetis) and Xylazine (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 \pm 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 A β 40.

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 A β 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.

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Figure legends

Fig. 1. A β 40 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 μ g/ml) or A β 40 (20 μ M) for 120 sec. In lane 4, as indicated, cultures were pretreated with the integrin $\alpha_{IIb}\beta_3$ antibody abciximab (0.5 μ g 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 μ g/ml) or A β 40 (30 μ M). 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 μ g/ml) or A β 40 (20 μ M) for 30 or 120 sec. α Tubulin served as loading control; n=6-7 mice per group.

Fig. 2. Reduced ATP release of GPVI-deficient platelets in response to A β 40. (A and B) Representative ATP release curves (A) and analysis (B) in human platelets pretreated (gray traces) with losartan (100 μ M) for 20 min then stimulated with CRP (5 μ g/ml) or A β 40 (20 μ M). Data are mean \pm SEM from n=5 donors; two-way ANOVA with Bonferroni's multiple comparison post hoc test: **p \leq 0.01; ***p \leq 0.001. (C) ATP release in WT and *Gp6*^{-/-} platelets

at rest, treated with CRP (5 $\mu\text{g/ml}$), A β 40 (10 μM), or A β 40 pretreated with integrin $\alpha_{\text{IIb}}\beta_3$ -blocking antibody Leo.H4 for 20 min. Data are mean \pm 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 A β 40. (A and B) Representative aggregation curves (A) and the quantified maximum aggregation of platelets treated with CRP (5 $\mu\text{g/ml}$) or A β 40 (20 μM) in the presence or absence of losartan (100 μM pretreatment for 20 min). Data are mean \pm 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 A β 40 (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 $\mu\text{g/ml}$) or A β 40 (10 μM). Data are mean \pm 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 A β 40 (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 , $n=5$ experiments. **(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 $\alpha_{\text{IIb}}\beta_3$ -blocking antibody Leo.H4 (each at 6 μg per 2 per 2*10⁶ cells). Scale bar, 50 μm , $n=5$ experiments. **(C and D)** Corresponding Western blotting and quantification of soluble A β in supernatants from murine platelets cultured as in (B). Leo.H4-treated platelets served as control. Data are mean \pm SEM from $n=5$ experiments; Students t-test, * $p \leq 0.05$. **(E)** Congo red staining of amyloid aggregates in cultures of platelets from WT and *Gp6*^{-/-} mice without A β 40 and with A β 40 (5 μM) in the presence or absence of Leo.H4 (6 μg per per 2 per 2*10⁶ cells). Scale bar, 50 μm , $n=5$ mice per group. **(F and G)** Representative Western blots (F) and quantification (G) of soluble A β in supernatants from WT and GPVI^{-/-} murine platelet cultures of remaining soluble A β . Controls lacking A β 40 (lane 1 in the blot) were not regarded in the analysis. Data are mean \pm SEM from $n=5$ mice per group two-way ANOVA with Bonferroni's post hoc test, * $p < 0.05$.

Fig. 5. A β 40 binds to GPVI. (A) Interaction screening using AVEXIS (Avidity-based Extracellular Interaction Screen). Biotinylated bait peptides A β 40 (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=5 experiments, 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 with A β 40 (20 μ M). Uncoated beads served as control. Immunoprecipitates were blotted against A β (6E10) and GPVI. Input = cell lysate. n=3 experiments. (D) Isolated platelets were stimulated with A β 40 (20 μ M) and immunoprecipitated with GPVI antibody. Immunoprecipitates were analyzed via Western blotting against A β and GPVI. N = 3. (E) WT and *Gp6*^{-/-} platelets were preincubated with A β 40 (5 μ M), followed by an incubation with anti-A β -FITC antibody. When indicated platelets were pretreated with integrin $\alpha_{IIb}\beta_3$ -blocking antibody Leo.H4. Binding of A β to platelet surface was measured by flow cytometry (n = 9-13 mice per group; mean \pm 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 A β and colocalization with amyloid aggregates in cell culture. (A) Western blotting of fibrinogen release in murine platelets upon stimulation with CRP (5 μ g/ml) or A β 40 (20 μ M) (pretreated as indicated with integrin $\alpha_{IIb}\beta_3$ blocking antibody Leo.H4 (0.5 μ g per 1 Mio cells) or with losartan (100 μ M)). 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 μ g per 1 Mio cells). Representative Western blot of n=3 experiments. (C) Murine platelets were incubated with A β 40 (5 μ M) 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 μ m. 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 μ g/ml) or A β 40 (20 μ M). 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 A β 40 (5 μ M) 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 A β (green). Scale bar, 20 μ m. Representative images of n=3 mice per group.

Fig. 7. Blocking or deletion of GPVI reduces A β -induced platelet adhesion in vivo. (A) Images of stable adherent and tethering A β 40-activated platelets 20 min after carotid artery ligation in vivo. WT platelets were either incubated only with A β 40 (top row) or were pretreated with losartan then incubated with A β 40 (middle row), and *Gp6^{-/-}* platelets were incubated with only A β 40. Carotid artery vessel wall is outlined using dotted lines. Arrows indicate adherent platelets. N=4-5 mice per group. Scale bar, 100 μ m. **(B and C)** Quantification of tethering **(B)** and stable adherent **(C)** A β 40-activated platelets. Data are mean \pm SEM; n = 4-5 mice per group. One-way ANOVA with Dunnett's post hoc test; *p \leq 0.05, **p \leq 0.01. **(D)** Tentative schematic illustration. Direct binding of A β 40 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 P2Y₁₂ receptor induces a shifting of integrin $\alpha_{IIb}\beta_3$ from a closed (inactive) to open (active) form leading to enhanced binding of A β to integrin $\alpha_{IIb}\beta_3$. Released fibrinogen bridges binding of soluble A β to GPVI and integrin $\alpha_{IIb}\beta_3$ to induce the formation of amyloid aggregates at the platelet surface ("4").