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## ORIGINAL ARTICLE

# Platelet glycoprotein VI-dependent thrombus stabilization is essential for the intraportal engraftment of pancreatic islets

Chunguang Chen<sup>1,2</sup> | Divya Rawat<sup>1</sup> | Balaji Samikannu<sup>1,3</sup> | Markus Bender<sup>4</sup> | Klaus T. Preissner<sup>5</sup> | Thomas Linn<sup>1</sup>

<sup>1</sup>Clinical Research Unit, Centre of Internal Medicine, Justus Liebig University, Giessen, Germany

<sup>2</sup>Paul Langerhans Institute Dresden (PLID) of the Helmholtz Zentrum München at the University Clinic Carl Gustav Carus of Technische Universität Dresden, Helmholtz Zentrum München, Neuherberg, Germany

<sup>3</sup>Cell and Developmental Biology, Weill Cornell Medicine Qatar, Doha, Qatar

<sup>4</sup>Institute of Experimental Biomedicine – Chair I, University Hospital and Rudolf Virchow Center, Würzburg, Germany

<sup>5</sup>Department of Biochemistry, Medical Faculty, Justus-Liebig-University, Giessen, Germany

## Correspondence

Thomas Linn, Clinical Research Unit, Centre of Internal Medicine, Justus Liebig University, Giessen, Germany.  
Email: Thomas.Linn@innere.med.uni-giessen.de

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Platelet activation and thrombus formation have been implicated to be detrimental for intraportal pancreatic islet transplants. The platelet-specific collagen receptor glycoprotein VI (GPVI) plays a key role in thrombosis through cellular activation and the subsequent release of secondary mediators. In aggregometry and in a microfluidic dynamic assay system modeling flow in the portal vein, pancreatic islets promoted platelet aggregation and triggered thrombus formation, respectively. While platelet GPVI deficiency did not affect the initiation of these events, it was found to destabilize platelet aggregates and thrombi in this process. Interestingly, while no major difference was detected in early thrombus formation after intraportal islet transplantation, genetic GPVI deficiency or acute anti-GPVI treatment led to an inferior graft survival and function in both syngeneic mouse islet transplantation and xenogeneic human islet transplantation models. These results demonstrate that platelet GPVI signaling is indispensable in stable thrombus formation induced by pancreatic islets. GPVI deficiency resulted in thrombus destabilization and inferior islet engraftment indicating that thrombus formation is necessary for a successful intraportal islet transplantation in which platelets are active modulators.

## KEYWORDS

basic (laboratory) research / science, coagulation and hemostasis, graft survival, islet transplantation, molecular biology

## 1 | INTRODUCTION

Pancreatic islet transplantation is a minimally invasive therapy to functionally cure type 1 diabetes and its complications. Islets are isolated from a deceased human donor pancreas and infused into a diabetic

patient's liver via the portal vein. The goal is to provide a sufficient number of insulin releasing  $\beta$ -cells to control blood glucose levels. Although the introduction of standardized protocols of islet isolation and transplantation has improved the clinical outcome it still suffers from low efficiency and adverse effects as not all recipients maintain

**Abbreviations:** IBMIR, instant blood-mediated inflammatory reactions; GPVI, glycoprotein VI; FcR $\gamma$ , Fc-receptor- $\gamma$  chain; Gp6<sup>-/-</sup>, GPVI knockout; WT, wild-type.

Chunguang Chen and Divya Rawat contributed equally to the study.

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long-term insulin independence and some experience graft failure.<sup>1,2</sup> Instant blood-mediated inflammatory reactions (IBMIR), which occur rapidly when isolated islets are infused to the portal vein and get direct contact with blood, have been suggested to be responsible for acute islet destruction observed after transplantation. IBMIR are clinically manifested by the formation of macro- and microthrombi and characterized by activation of platelets and the coagulation system.<sup>1,3,4</sup> While platelet consumption and formation of a fibrin capsule around the islet is known to be one of the first events in IBMIR, the molecular mechanisms behind platelet activation and their contribution to islet engraftment remain largely unknown.

Platelets are important players in hemostasis by sealing vascular lesions to prevent bleeding and wound infection. Adhesion of platelets to the extracellular matrix (ECM) of the exposed vessel is initiated by the interaction between glycoprotein (GP)Ib/V/IX receptor complex and von Willebrand factor (vWF) as well as between platelet GPVI/GPIa and collagen at sites of vascular injury.<sup>5</sup> Although the binding of multimeric vWF to GPIb/V/IX is required for the initial adhesion of platelets under high shear conditions, this interaction is insufficient for stable adhesion.<sup>6</sup> GPVI, a 60–65 kDa type I transmembrane receptor belonging to the immunoglobulin superfamily, is noncovalently linked to a homodimer of the Fc-receptor- $\gamma$  chain (FcR $\gamma$ ), and known to be a major platelet collagen adhesion receptor.<sup>7</sup> Binding of GPVI to fibrillar collagen initiates platelet cellular activation followed by converting the integrins GPIa/IIa and GPIIb/IIIa into high-affinity adhesion receptors required for stabilizing platelet aggregation. Platelet GPVI can also be activated by other ligands of the ECM, including laminin and fibrin.<sup>8–10</sup> The concomitant release of second-wave agonists, most notably ADP, ATP, and TxA<sub>2</sub>, will amplify integrin activation on adherent platelets and mediates platelet plug growth by activating additional bystander platelets.<sup>11</sup> Thus, GPVI-mediated platelet signaling has been shown to be a prerequisite for efficient platelet adhesion, aggregation, degranulation, and procoagulant activity,<sup>12</sup> whereby GPVI has been proposed to be a central player in platelet activation and thrombus formation.<sup>6</sup>

Collagens and noncollagenous glycoproteins such as fibronectin and laminin are present in the extracellular matrix and the basement membrane of pancreatic islets.<sup>13–17</sup> They are involved in the regulation of islet cell differentiation partly by integrin-dependent signaling, particularly enabling  $\beta$ -cells to form clusters.<sup>18,19</sup> The vessel wall ECM-associated adhesive proteins are normally shielded from circulating blood due to their localization in the basement membrane; however, they would get into direct contact with blood when infused into the portal vein.<sup>20</sup> While tissue factor (TF) expressed in islet endocrine cells was found to trigger IBMIR and anti-TF treatment to suppress IBMIR and early graft loss,<sup>21–25</sup> we hypothesized that platelets and platelet GPVI signaling could be active players in IBMIR and islet engraftment because they could be activated via interactions between platelet GPVI and islet surface glycoproteins, including collagen, laminins and fibrin, and as such modulate thrombotic events in IBMIR and the islet transplantation outcome.

In the current study, we examined the role of platelet GPVI signaling in islet-mediated thrombotic reactions in the context of IBMIR

and its contribution to intraportal islet engraftment outcome. The results demonstrate that GPVI-dependent platelet activation and thrombus stabilization are required for proper outcome of pancreatic islet transplantation.

## 2 | RESEARCH DESIGN AND METHODS

### 2.1 | Animals

Fourteen-week-old male C57BL/6 N wild-type (WT) mice and 10-week-old male NMRI nu/nu mice were purchased from Charles River. GPVI knockout (*Gp6<sup>-/-</sup>*) mice used in this study had been described in previous studies.<sup>26,27</sup> FcR $\gamma$  chain mutated mice with C57BL/6 background were kindly provided by J. Leusen (Utrecht, Netherlands).

### 2.2 | Islet isolation

All the islet isolations were performed at the Islet Isolation Facility of Clinical Research Unit (Justus Liebig University). Human islets were isolated from a single donor pancreas using previously described techniques of collagenase digestion and Ficoll purification.<sup>28</sup> Porcine islets were isolated as previously described.<sup>29,30</sup> Murine islets were isolated from C57BL/6 mice using pancreatic tissue distension with collagenase through common bile duct. Following digestion, mouse islets were purified by hand picking. After isolation, the quality of islets was evaluated by trypan blue exclusion, dithizone staining, and microscopic inspection. Both purity and viability of islets were more than 90% for subsequent experiments. The islets were cultivated in a humidified air atmosphere following isolation and used within 7 days.

For preparation of pseudo-islets, MIN-6 (mouse insulinoma, 6th subclone) cells were cultured, washed with Dulbecco's phosphate buffered saline (PBS) (without Ca<sup>2+</sup> and Mg<sup>2+</sup>) and treated with trypsin/(ethylenediaminetetraacetic acid) EDTA solution. After incubation for 2–3 min at 37°C, the detached cells were washed with warm Panserin 401 and cultivated overnight for formation of pseudo-islets.

### 2.3 | Platelet aggregometry

Blood was drawn from C57BL/6 N wild-type (WT) and GPVI knockout (KO) mice into a heparin (20 U/ml)-containing tube and blood was centrifuged at 800 rpm for 6 min at room temperature. Supernatant was collected and platelet-rich plasma (PRP) was obtained. PRP was diluted 1:3 in Tyrode's buffer for platelet aggregation assay where aggregation was induced by 2 or 10  $\mu$ M ADP. Light transmission was recorded by Fibrinometer 4-channel aggregometer (APACT Laborgeräte und Analysensysteme) over 10 min. Untreated plasma was diluted 1:3 and set as 100% aggregation for calibration.

## 2.4 | Ex vivo dynamic thrombus formation assay

Thrombus formation in whole blood was monitored under flow conditions using the BioFlux 200 system including inverted microscope and CCD camera (I&L instruments, Germany). A 48-well plate with 24 channels was coated with 100  $\mu\text{g}/\text{ml}$  collagen I solution (Gibco, Invitrogen) per outlet well. C57BL/6 or *Gp6<sup>-/-</sup>* citrated blood (180  $\mu\text{l}$  each) which was labeled with fluorescent dye Calcein AM (ThermoFisher) together with 20 porcine islets was added to the channels. After the addition of ADP (2.5  $\mu\text{M}$ ) into both channels, perfusion at 2  $\text{dyn}/\text{cm}^2$  was immediately started. During the course of the flow experiment, images of the channels were recorded whereby the position of the camera was not changed. Recorded images were exported into ImageJ to analyze the clot area. Clot areas were measured in every image to receive mean and standard deviation for one run per channel. The time profile of clot area change was subjected to regression analysis. Linear model resulted in slope of aggregation starting at basal up to time point of maximum clot area. Accordingly, disaggregation was defined as declining clot area from maximum back to basal. If time point zero basal clot area was not attained in disaggregation phase during observation time, the last measured value was carried forward.

## 2.5 | Intraportal islet transplantation

Intraportal islet transplantation was performed using WT C57BL/6, Fc $\gamma$ -deficient, *Gp6<sup>-/-</sup>* mice, or NMRI nu/nu mice as recipients. Before transplantation, diabetes was induced in the recipients by a single intraperitoneal injection of 180  $\text{mg}/\text{kg}$  streptozotocin, and nonfasting blood glucose levels were measured from the tail vein using a glucometer (Elite, Bayer). Mice with a blood glucose value higher than 300  $\text{mg}/\text{dl}$  for at least 2 consecutive days were used as recipients. About 300 mouse islets each were transplanted into the liver of recipients via the portal vein with a 27-gauge needle using a previously described method with modifications.<sup>31</sup> Approximately 2,000 human islet equivalents (IEQ) were transplanted to male NMRI nu/nu mice using the same technique. In order to deplete GPVI from circulating platelets,<sup>32</sup> 50  $\mu\text{g}$  of anti-GPVI monoclonal antibody (mAb) JAQ1 (Rat IgG2a) (Emfret, Würzburg, Germany) or isotypic immunoglobulin as a control (Origene, Herford, Germany) was injected intraperitoneally into NMRI nu/nu mice 60 min before transplantation. During surgery, the animals were anesthetized using 2.5% avertin (200  $\mu\text{l}/100$  g) (Sigma) and maintained with isoflurane. The wound was closed in two layers with absorbable sutures. Euglycemia and graft survival were defined as nonfasting blood glucose with less than 200  $\text{mg}/\text{dl}$  on 3 consecutive days.

## 2.6 | Immunohistochemistry

Grafted livers or blood islet clots were recovered and snap-frozen in O.C.T medium (Sakura). Five-micrometer sections were cut and mounted onto SuperFrost Ultra Plus slides, air dried and fixed in

Zamboni fixative for 10 min before staining. After blocking in PBS with 1% BSA and 2% donkey serum, the cryosections were probed with antibodies against GPIIb $\alpha$  (pop3/4), fibrinogen (AbD Serotec), CD11b (M1/70.15, ImmunoTools), or insulin (GPAIS, Dako) at 4°C overnight. Secondary fluorescence dye-coupled antibodies (Jackson ImmunoResearch) were applied on the next day. Nuclei were visualized by Hoechst 33342 (Sigma). The cover slips were mounted with ProlongGold (Invitrogen, Darmstadt, Germany), visualized and photographed using a Leica DMLB microscope (Leica, Germany) equipped with Leica DFC420C CCD and processed in Leica Application Suite.

## 2.7 | Statistics

All results are expressed as mean  $\pm$ SEM. Statistical significance was determined using student's *t*-test or one-way ANOVA or two-way RM ANOVA, as appropriate. Graft survival between groups was analyzed by Logrank (Mantel-Cox test) and Gehan-Breslow-Wilcoxon test. Rate and maximum disaggregation of platelets were defined by slope and minimum of aggregation curve and calculated by fitting with linear equation. All statistical analyses were performed with GraphPad Prism (version 8.3.0, GraphPad Software; San Diego, USA). A value of  $p < .05$  was considered statistically significant.

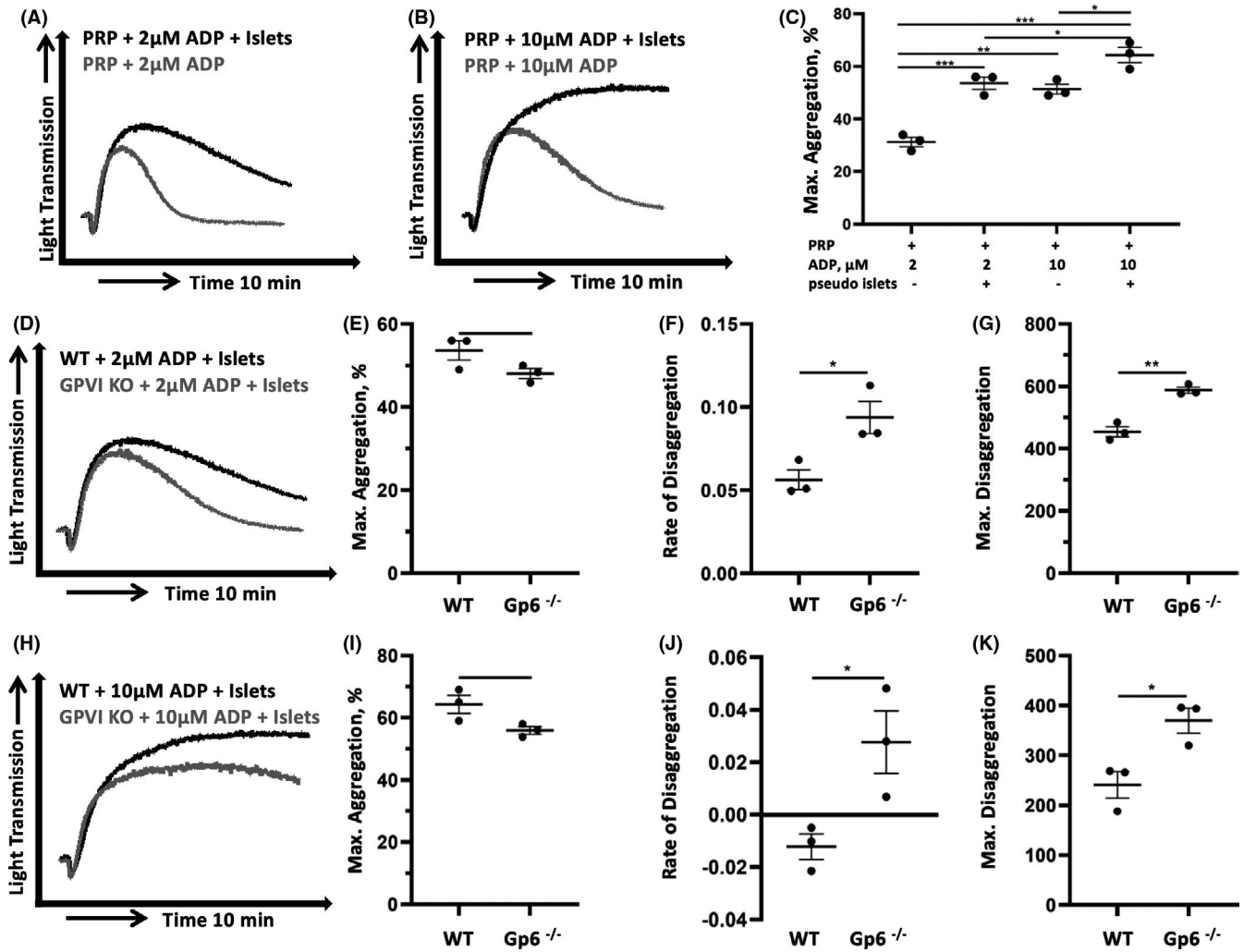
## 3 | RESULTS

### 3.1 | Platelet GPVI deficiency facilitates platelet disaggregation

Platelet aggregation was investigated in vitro by aggregometry using mouse PRP. ADP dose-dependently induced platelet aggregation in PRP from WT mice as expected. ADP-induced platelet aggregation was further promoted by mouse pseudo-islets (Figure 1A-C) or isolated porcine islets (Figure S1) such that the graphical record of aggregation reached higher peak values and was more persistent. A different aggregation profile was observed when PRP from *Gp6<sup>-/-</sup>* mice was used (Figure 1). Although both the rate and the maximum of aggregation were not different as compared to the WT (Figure 1), the aggregates of GPVI-deficient platelets were less stable and disaggregated faster. With the presence of pseudo-islets at both ADP concentrations, the rate (Figure 1) and maximal disaggregation (Figure 1) were significantly higher in PRP from *Gp6<sup>-/-</sup>* mice as compared to WT mice.

### 3.2 | GPVI deficiency destabilizes thrombus formation

*Ex vivo* thrombus formation in whole blood was monitored in a microfluidic device. In WT mouse blood, ADP stimulation induced the formation of small and nonadherent thrombi in the absence of pancreatic islets (Figure 2A WT- islets and Figure 2B; maximal clot area  $381 \pm 181 \mu\text{m}^2$ ), whereas large and adherent thrombi were observed



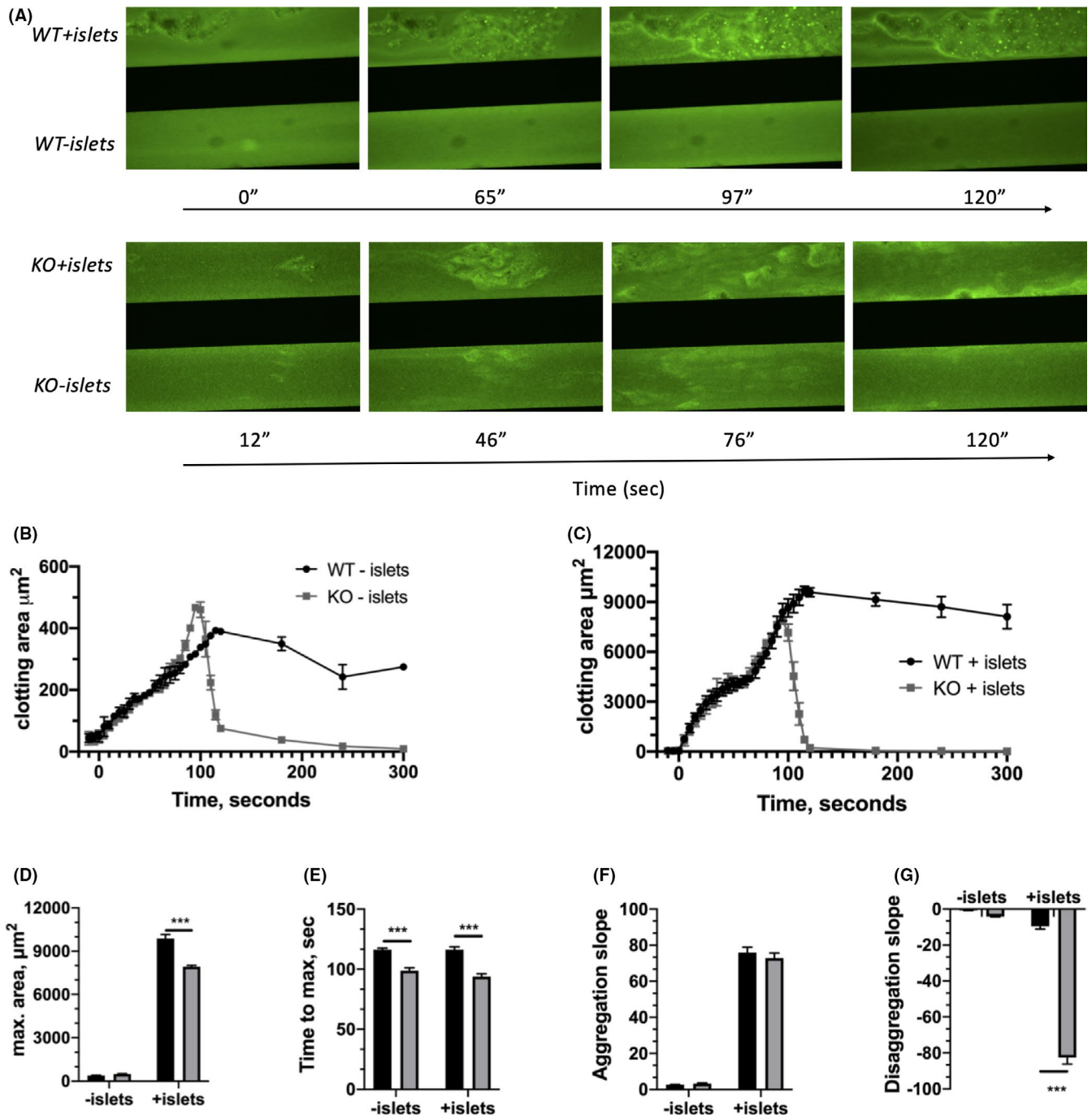
**FIGURE 1** Platelet GPVI deficiency destabilizes platelet aggregation. In platelet-rich plasma (PRP), platelet aggregation was induced by 2 μM (A) or 10 μM (B) ADP in the absence or presence of pseudo-islets, and light transmission was followed. The maximum degree of platelet aggregation in PRP from WT mice was quantified for the indicated conditions (C). Platelet aggregation was induced by 2 μM (D to G) or 10 μM (H to K) ADP in PRP from WT or GPVI knockout (*Gp6*<sup>-/-</sup>) mice, and the extent of aggregation was quantified by light transmission. The maximum degree of aggregation (E, I), the rate of disaggregation (F, J), and the maximal disaggregation (G, K) were calculated. \**p* < .05, \*\**p* < .01, and \*\*\**p* < .001 vs indicated comparator

in the presence of porcine islets (Figure 2A WT+islets and Figure 2C; maximal clot area  $9433 \pm 1045 \mu\text{m}^2$ ). Thrombus formation in the blood from *Gp6*<sup>-/-</sup> mice was faster (Figure 2B,E); however, it was smaller in the presence of porcine islets (Figure 2C,D). While the rates of aggregation (Figure 2F) were not different between groups, rates of thrombus disaggregation (Figure 2G) were significantly faster in blood from *Gp6*<sup>-/-</sup> mice as opposed to WT, indicating that the thrombi formed in the former group were substantially less stable.

### 3.3 | GPVI/FcR $\gamma$ deficiency does not impair early thrombus formation but compromises engraftment of syngeneic mouse islets

Syngeneic islet transplantations were performed to investigate the contribution of platelet GPVI/FcR $\gamma$  signaling after intraportal islet

transplantation. Isolated WT mouse islets were transplanted into WT mice, or GPVI-deficient mice in either FcR $\gamma$ -mutant mice or induced by anti-GPVI treatment with a monoclonal antibody (mAb) JAQ1. JAQ1 treatment has been shown to induce a rapid and specific depletion of the collagen receptor GPVI from circulating platelets and offer a long-term antithrombotic protection in the treated mice.<sup>33</sup> The grafted livers were recovered at 4 h after transplantation for IHC investigations on the early posttransplant IBMIR events. The transplanted islets were entrapped with platelets and fibrin in the WT mouse liver after transplantation (Figure 3A,D). Leukocyte infiltration toward the islet graft was evidenced by CD11b immunostaining (Figure 3G). Interestingly, no major differences in the staining patterns of platelets or CD11b<sup>+</sup> cell infiltration was observed in these early grafted livers in GPVI-deficient recipient mice as compared to WT. Similar to the WT liver, thrombus formation and CD11b<sup>+</sup> leukocyte accumulation could be readily detected in

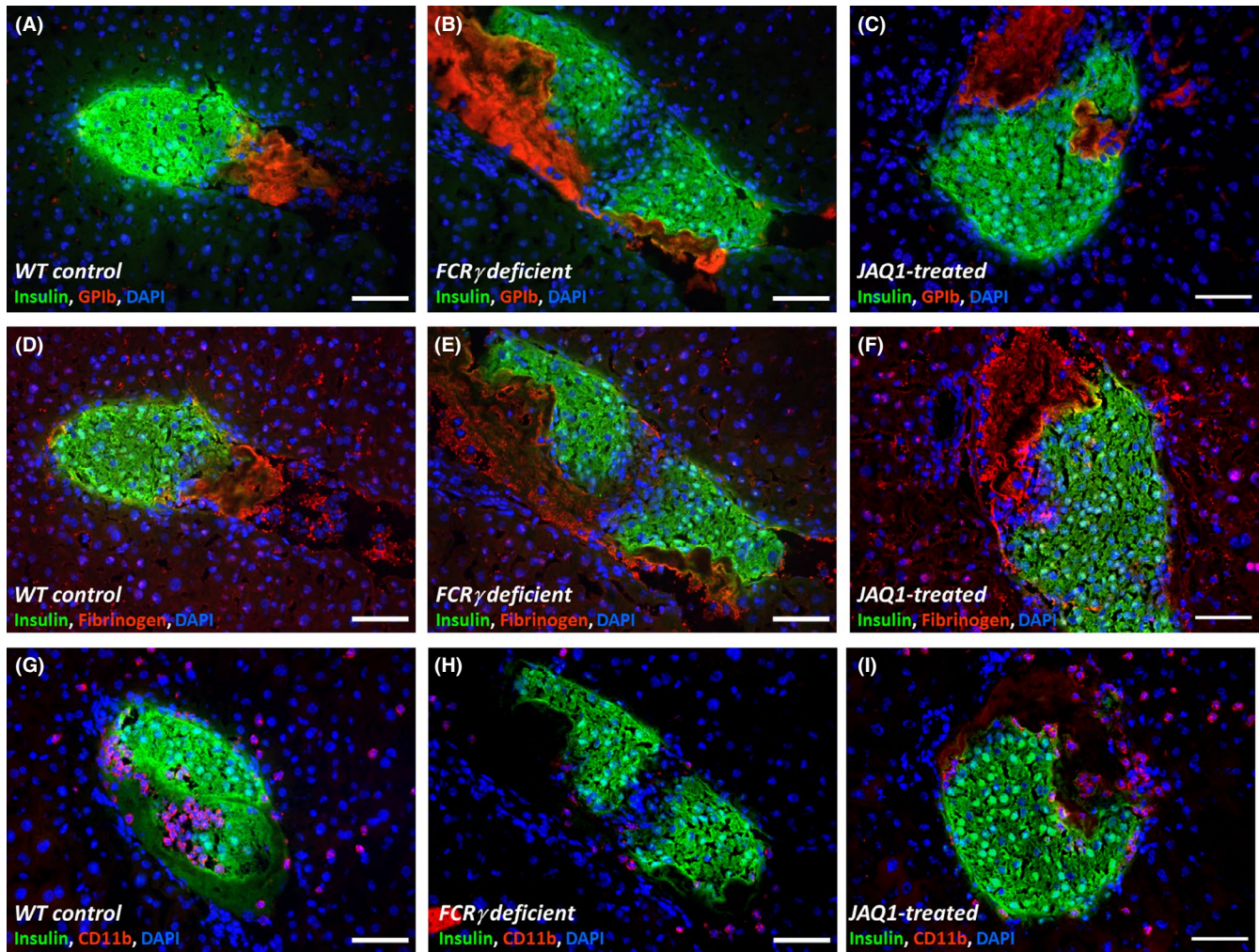


**FIGURE 2** Platelet GPVI deficiency reduces the stability of islet-induced thrombus formation ex vivo. Representative images of thrombus formation in a microfluidic device using wild-type (A, WT) or *Gp6*<sup>-/-</sup> (A, KO) mouse blood are shown in dual flow 24-well plates. Top inlet tube is represented by upper green area and carries blood with pancreatic islets (+ islets). Bottom inlet is separated from top tube conveying blood without pancreatic islets (- islets). Thrombus formation was initiated with 2  $\mu$ M ADP within a time lapse of 2 min between the images from left to right shear of 2 dyn/cm<sup>2</sup>. The time profiles of thrombus formation are shown (B and C) where the clotting areas were calculated from two-dimensional images taken every 5 s. The maximum thrombus area (D), t Time to maximum thrombus formation (E), the aggregation slope (F), and disaggregation slope (G) were calculated from WT (black circles) and *Gp6*<sup>-/-</sup> (gray squares) blood. *n* = 4 runs. \*\*\* *p* < .001 vs WT; two-way ANOVA

the liver sections from either FcR $\gamma$ -mutant mice (Figure 3B,E,H) or JAQ1-treated mice (Figure 3C,F,I) after islet transplantation.

To investigate the contribution of platelet GPVI/FcR $\gamma$  signaling in islet engraftment, syngenic islet transplantations were performed where diabetic mice were used as recipients. The islet transplants

resumed euglycemic control in 100% (5/5) WT mice mostly (4/5) within 1 day after transplantation and latest (1/5) within 5 days (Figure 4A,B). In contrast, only 60% (3/5) of the FcR $\gamma$ -deficient mice turned euglycemic with the same number of transplanted islets, and these mice needed more time (3 to 7 days) to resume euglycemic



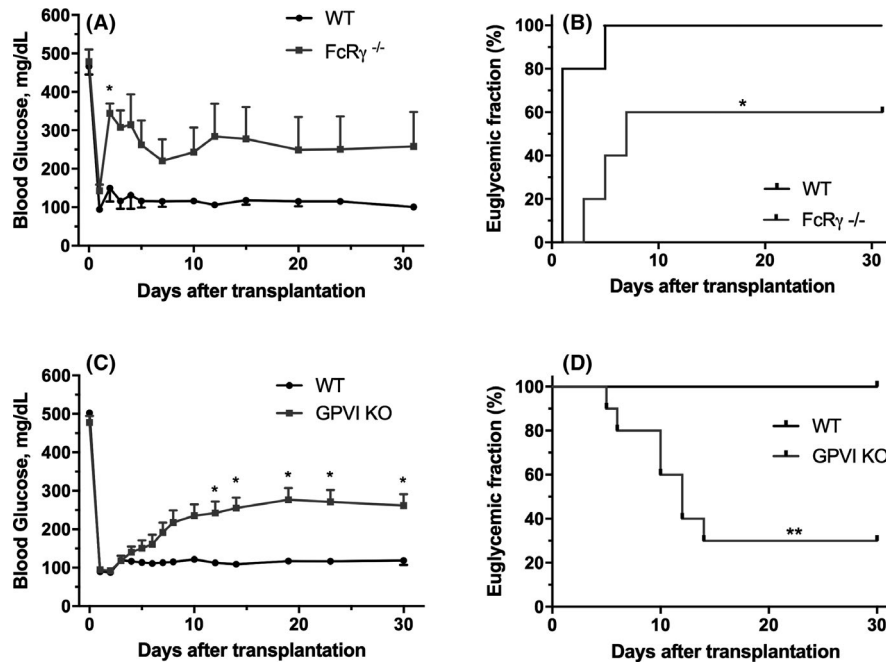
**FIGURE 3** Immunohistochemical staining of grafted liver sections. Cryosections from grafted liver from WT (A, D, G), FcR $\gamma$ -deficient (B, E, H), or JAQ1-treated (C, F, I) recipient mice, recovered 4 h after transplantation, were probed for insulin (green) and GPIIb $\alpha$  (red) for platelet aggregations (A-C); or for insulin (green) and fibrinogen (red) for fibrin formation (D-F); or for insulin (green) and CD11<sup>b</sup> (red) for leukocyte infiltration (G-I); scale bar=50  $\mu$ m

control. Logrank analysis confirmed a significant disadvantage of FcR $\gamma$  deficiency (chi-square 5.663,  $p = .0173$ ; Figure 4B) under such conditions. Similar results were obtained when Gp6<sup>-/-</sup> mice were used as recipients in a separated experiment. In contrast to the WT mice which resumed immediate and stable euglycemia after islet transplantation, Gp6<sup>-/-</sup> recipients lost euglycemic control within a short period of time (Figure 4C; graft survival time  $16.6 \pm 3.7$  days,  $p < .001$ ). Logrank analysis confirmed a significant disadvantage of GP6<sup>-/-</sup> mice (chi-square 13.31,  $p = .0003$ ; Figure 4D) for this maneuver.

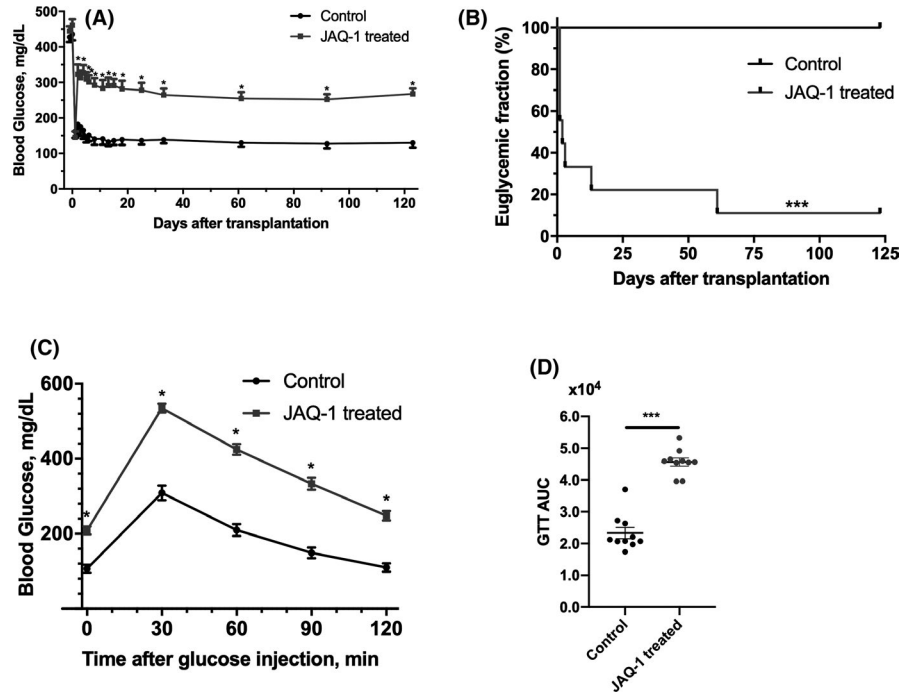
### 3.4 | Anti-GPVI treatment compromises xenogeneic human islet engraftment

The roles of platelet GPVI signaling in human islet engraftment was studied by injecting an anti-GPVI monoclonal antibody (mAb) JAQ1 into diabetic NMRI nu/nu recipient mice to specially deplete

GPVI from circulating platelets before human islet transplantation. Consistent with the syngeneic mice islet transplantation data, acute GPVI depletion in circulation platelets with a single bolus JAQ1 injection before transplantation led to an inferior islet transplantation outcome. Using almost identical islet mass transplanted ( $2286 \pm 48$  IEQ in control group vs  $2294 \pm 36$  IEQ in JAQ1-treated group, n.s.), whereby all control mice resumed an almost immediate and long-term stable euglycemia after transplantation, JAQ1-treated recipient mice became hyperglycemic again within 2 to 4 days after transplantation (median graft survival time; 2 days) (Figure 5A). Logrank analysis confirmed a significant disadvantage of anti-GPVI treatment (chi-square 15.00,  $p = .0001$ ; Figure 5B). An intraperitoneal glucose tolerance test (IPGTT) was performed on all recipient mice by the end of the study. Compared to the control mice, JAQ1-treated mice had significantly higher blood glucose at each time point (Figure 5C), a significantly higher area under the curve (Figure 5D), and lower insulin secretion (Figure S4), further confirming the impaired graft function after anti-GPVI treatment.



**FIGURE 4** GPVI/FcR $\gamma$  deficiency impairs syngeneic mouse islet engraftment. Streptozocin-induced diabetic mice were transplanted with syngeneic islets intraportally. Mean nonfasting blood glucose levels (A) are plotted and euglycemia fraction curves (B) are shown for FcR $\gamma$ -deficient or WT control recipients, respectively ( $n = 5$  each). Mean nonfasting blood glucose levels (C) are plotted and euglycemia fraction curves (D) are shown for GPVI knockout (black triangle) or WT control (black square) recipients, respectively ( $n = 10$  each). \* $p < .05$  vs. WT; \*\* $p < .01$  vs WT



**FIGURE 5** GPVI/FcR $\gamma$  deficiency impairs xenogeneic human islet engraftment. Streptozocin-induced diabetic NMRI nu/nu mice were transplanted with human islets intraportally. Mean nonfasting blood glucose levels (A) are plotted and euglycemia fraction curves (B) are shown for JAQ1-treated or control recipients, respectively ( $n = 10$  each). At the end of the experiment, an intraperitoneal glucose tolerance test (2 g/kg glucose i.p.) was performed using collecting blood at -10, 0, 15, 30, 45, 60, 90, and 120 min (C) after 16-h fasting. Area under the glucose curve during IPGTT was calculated and plotted (D). \* $p < .05$  vs WT; \*\*\* $p < .001$  vs WT



## 4 | DISCUSSION

In the current study, the influence of platelet aggregation and thrombus formation on the efficacy of intraportal islet engraftment was investigated with particular emphasis on the role of GPVI/FcR $\gamma$ , the prominent platelet receptor for collagen and other matrix proteins. When isolated islets are infused into the portal vein and get in direct contact with different blood constituents, platelet-dependent hemostasis and the blood coagulation system are triggered intravascularly.<sup>1-4,34</sup> From a mechanistic angle, however, the contribution of key adhesion receptors of platelets in these events has never been explored.

Thrombus formation triggered by intravascularly infused islets in direct contact with blood was believed to be dependent on the extrinsic tissue factor (TF) pathway. Tissue factor was found to be synthesized and secreted by pancreatic islets, and blocking the active site of TF with specific antibodies or inactive factor VIIa had been found to inhibit the thrombosis reactions triggered by islets in vitro.<sup>21</sup> In addition, TF gene knockdown in porcine islets had been shown to suppress IBMIR and reduced neutrophil infiltration in a looping system.<sup>24,25</sup> In a recent study, it was shown that expression of TF in islets and  $\beta$ -cells was induced by cytokines (IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ ) and that TF/FVIIa signaling promoted cytokine-induced  $\beta$ -cell death.<sup>35</sup> Furthermore, melagatran, a specific thrombin inhibitor, was reported to partly reduce IBMIR.<sup>36</sup> However, blockage of this pathway by either melagatran or heparin could not completely prevent the activation of platelets and the formation of fibrin surrounding the transplanted islets or the infiltration of CD11b<sup>+</sup> cells,<sup>20,36</sup> indicating other pathways to be involved in parallel.

We investigated the contribution of platelet GPVI signaling in the thrombosis events triggered by pancreatic islets at different complexity levels in this study: firstly, using in vitro platelet aggregation with mouse PRP, pancreatic islets profoundly promoted ADP-induced platelet aggregation. Compared to the aggregates formed in PRP from WT mice (while the rate and the maximum of aggregation were not different in the initiating phase), islet-promoted platelet aggregates in PRP from *Gp6*<sup>-/-</sup> mice were less stable and disaggregated much faster. Secondly, ex vivo thrombus formation was examined in a microfluidic device, thereby modeling blood flow in the portal vein where stable thrombus formation was triggered by pancreatic islets when using whole blood from WT mice. While initial thrombus formation was not much different between groups and consistent with the platelet aggregation data, in blood from *GP6*<sup>-/-</sup> mice the thrombi were unstable and disaggregated at a significantly higher rate as compared to control. Similar results were obtained when human islets, instead of porcine islets, were used to induced thrombus formation in mouse blood (Figure S2; Table S1). Together, these data indicate that, while it might not be required for the initial phase of platelet aggregation and thrombus formation promoted by pancreatic islets, platelet GPVI signaling appears to be essential for the stability of platelet aggregates and subsequent thrombus formation.

Next, the relevance of these in vitro and ex vivo results for in vivo thrombus formation and leukocyte recruitment were

investigated in a model of intraportal islet transplantation. Interestingly, we found no major difference in the staining pattern of formed platelet plugs surrounding the transplanted islets or the infiltration of CD11b<sup>+</sup> cells in the early 4-h recovered liver from GPVI/FcR $\gamma$ -deficient recipient mice as compared to the WT mice. PSGL-1 and CD11b staining on 24-h recovered livers also showed a similar degree of leukocyte accumulation surrounding the islet grafts in JAQ1-treated or control mice (Figure S3). The lack of differences in thrombus formation in vivo between the WT and GPVI-deficient recipients could indicate a different kinetics of thrombus formation and dissociation in the hepatic sinusoids of the portal vein system as compared to the in vitro or ex vivo situations. More importantly, since no heparin was administered to these mice during transplantation, our data could result from the possibility that TF-thrombin pathway was predominant over GPVI/FcR $\gamma$  pathway in the in vivo situation. Indeed, although GPVI has been shown to potentiate thrombin-induced platelet activation,<sup>37,38</sup> thrombin could effectively overcome GPVI/FcR $\gamma$  deficiency.<sup>39,40</sup> Our immunohistochemical analysis also indicates that GPVI signaling might not be required for the initiation of thrombus formation induced by infused islets which is consistent with our in vitro and ex vivo data where the initiation of platelet aggregation as well as thrombus formation was largely unaffected by GPVI deficiency. Finally, the contribution of platelet GPVI/FcR $\gamma$  pathway in islet engraftment was investigated in two different transplantation models. Here, delayed engraftment or earlier graft failure was independently observed in two strains of recipient mice genetically deficient for GPVI/FcR $\gamma$  in a syngeneic islet transplantation model. Similarly, an acute anti-GPVI treatment with a single bolus JAQ1 antibody injection in NMRI nu/nu mice resulted in an immediate and long-term impairment in graft function model in a xenogeneic human islet transplantation model.

While thrombus formation was suggested to be associated with islet damages and low transplantation efficiency,<sup>20,36</sup> our results imply GPVI-dependent platelet activation and stable thrombus formation in the early posttransplant phase to be necessary for successful islet engraftment and long-term graft function (Figure S7).

Platelets could potentially modulate islet engraftment by several mechanisms that remain to be explored in future studies. For example, they have been shown to contain a considerable amount of TF in its inactive form which could be transformed into its active form and translocate onto the platelet surface upon collagen activation and interaction with neutrophil or monocytes.<sup>41</sup> At the same time, platelets are known to contain tissue factor pathway inhibitor (TFPI) which is a physiological antagonist of the TF pathway and could be rapidly released from platelets upon activation.<sup>42</sup> Thus, platelets can modulate IBMIR by balancing the TF/TFPI system also during their interaction with the islet-associated TF pathway. Moreover, platelets contain and release matrix metalloproteinases (MMPs) and their inhibitors that could modulate thrombus formation as well.<sup>43,44</sup> In return, these proteases can differently regulate platelet activation and functions, including GPVI signaling.<sup>45-48</sup> In

particular, our group has previously shown that MMP-9 expression and activity, which are associated with platelets and granulocytes, were found to be increased after transplantation, whereas inhibition of MMPs reduced leukocytes infiltration and improved islet transplantation outcome.<sup>49</sup> In addition, MMP-9, which could be delivered by a subset of neutrophils recruited to the transplantation site, was shown to be required for islet graft revascularization and functional integration.<sup>50</sup> These findings suggest that platelets may modulate transplantation outcome by governing protease activity directly into the locally concentrated engrafted site. Furthermore, platelets are known to secrete a variety of proteins upon activation, including growth factors and chemokines.<sup>51</sup> Together, these platelet-associated activities can influence different steps and components of intraportal islet engraftment, including the acute thrombus reactions as well as islet revascularization and function in the long term.

Besides its roles in thrombus formation and stability, the induction of which by isolated islets had been investigated in this study, platelet GPVI has been recently also suggested to be an immune receptor to play an active role in the cross talk between inflammatory and thrombotic pathomechanisms.<sup>52</sup> In addition to collagen, GPVI could be activated by numerous other ligands, including fibrin, fibrinogen, and the membrane protein EMMPRIN. Blockage of GPVI has been shown to affect immune cell recruitment, activation, and function at the inflammation site in different disease models.<sup>53-56</sup> Since islet infusion into the portal vein system induced a cascade of events, including both thrombus formation and inflammation (thrombo-inflammation), our study is limited to provide a mechanistic insight into the interactions between infused islet and different components of the portal vein blood together with the local microenvironment that collectively determines the transplantation outcome. How different platelet activities are coordinated in this respect and how their interactions with various blood cells, the temporal dynamics and their contribution to islet engraftment are regulated, warrant more investigations.

In summary, we here show that even though it might not be required for the initiation of platelet aggregation and thrombus formation in vitro and in vivo, platelet GPVI/Fc $\gamma$  signaling is essential for the stability of platelet aggregates and formed thrombi as well as for the engraftment and function of the transplanted islets. Therefore, our data indicate for the first time that thrombus formation, triggered by the infused islets, is required and potentially beneficial for the successful intraportal islet engraftment in which platelet are active modulators.

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#### DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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