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Mice expressing low levels of CalDAG-GEFI exhibit markedly impaired platelet activation with minor impact on hemostasis

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Abstract

Objective—The tight regulation of platelet adhesiveness, mediated by the αIIbβ3 integrin, is critical for hemostasis and prevention of thrombosis. We recently demonstrated that integrin affinity in platelets is controlled by the guanine nucleotide exchange factor, CalDAG-GEFI (CD-GEFI), and its target, RAP1. In this study, we investigated whether low-level expression of CD-GEFI leads to protection from thrombosis *without* pathological bleeding in mice.

Approach and Results—*Cdg1*^{low} mice were generated by knock-in of human CD-GEFI cDNA into the mouse *Cdg1* locus. CD-GEFI expression in platelets from *Cdg1*^{low} mice was reduced by ~90% when compared to controls. Activation of RAP1 and αIIbβ3 was abolished at low agonist concentrations and partially inhibited at high agonist concentrations in *Cdg1*^{low} platelets. Consistently, the aggregation response of *Cdg1*^{low} platelets was weaker than that of wild-type (*WT*) platelets, but more efficient than that observed in *Cdg1*$^{-/-}$ platelets. Importantly, *Cdg1*^{low} mice were strongly protected from arterial and immune complex-mediated thrombosis, with only minimal impact on primary hemostasis.

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DISCLOSURES

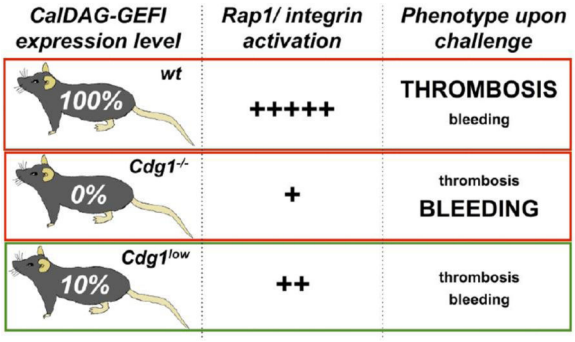
D.O.C. is employed by, has equity ownership in and serves on the board of directors of TransViragen, the company which has been contracted by UNC-Chapel Hill to manage its Animal Models Core Facility. The other authors declare no competing financial interests.

AUTHORSHIP

Contribution: R.P., and D.S.P, designed and performed experiments, analyzed and interpreted data, and wrote the paper; R.H.L. and B.C.C. performed experiments; S.E.M. helped design, interpret and write up experiments on immune-mediated thrombosis and thrombocytopenia; L.V.P. assisted with data interpretation and the writing of the manuscript; D.O.C. designed the *Cdg1*^{low} allele and supervised generation of the mice by the Animal Models Core staff; W.B. designed experiments, analyzed and interpreted data, and wrote the paper; and all authors critically reviewed the manuscript.

Conclusion—Together, our studies suggest the partial inhibition of CD-GEFI function as a powerful new approach to safely prevent thrombotic complications.

GRAPHICAL ABSTRACT



Keywords

Platelet; Hemostasis; Thrombosis; Mouse

INTRODUCTION

Platelet aggregate formation, mediated by activated integrin αIIbβ3, is critical for hemostasis upon tissue injury. Excessive platelet activation and aggregation, however, can lead to thrombotic complications, such as heart attack and stroke[1, 2]. The signaling events required for platelet integrin activation are initiated by engagement of surface-expressed receptors for agonists such as thrombin and/or collagen[3]. These agonists trigger a first wave of platelet signaling that includes a rapid increase in the cytosolic calcium concentration ($[Ca^{2+}]i$). This increase in $[Ca^{2+}]i$ leads to activation of the calcium-binding guanine nucleotide exchange factor, CalDAG-GEFI (CD-GEFI; RASGRP2), a key regulator of the small GTPase RAP1[4]. RAP1 controls various platelet responses important for hemostatic plug formation, including the inside-out activation of integrin receptors[5, 6]. In the absence of additional signals, CD-GEFI-mediated RAP1 signaling is terminated by the GTPase-activating protein (GAP), RASA3[7]. The signal for RASA3 inactivation and sustained RAP1 signaling is provided by stimulation via P2Y12, the receptor for the second-wave mediator ADP and the target for various clinically used anti-platelet drugs[8, 9].

Work by us and others provided important mechanistic information on how interference with RAP1 signaling affects platelet adhesion at sites of vascular injury. Mice deficient in the main RAP1 isoform, RAP1B, exhibit marked defects in platelet aggregation to various agonists *in vitro* and impaired hemostasis and thrombosis *in vivo*[10, 11]. As outlined above, the activity state of RAP1 in platelets is controlled by CD-GEFI and RASA3[4, 7]. Active Rasa3 is required to keep circulating platelets in a quiescent state[7]. During hemostatic plug formation, its activity must be down-modulated as part of the P2Y12/PI3 kinase signaling pathway in order for stable platelet adhesion to occur. Consistently, mice lacking RASA3 are characterized by severe thrombocytopenia due to platelet pre-activation and clearance[7], while both hemostatic plugs and pathological thrombi in mice lacking P2Y12 function are

highly unstable[9, 12]. In contrast, RAP1 activation in platelets from mice lacking CD-GEFI occurs with a delay and requires higher doses of strong agonists, such as thrombin and collagen. Under flow conditions *in vitro*, platelets lacking CD-GEFI (*Cdg1*$^{-/-}$) are markedly impaired in their ability to form three-dimensional thrombi, especially under conditions of high shear stress[12]. Consistent with the *in vitro* phenotype, *Cdg1*$^{-/-}$ mice are strongly protected from both immune-mediated thrombocytopenia and thrombosis (ITT) and arterial thrombosis, but they also show a marked defect in hemostatic plug formation[12, 13].

Importantly, the main findings in *Cdg1*$^{-/-}$ mice were recently confirmed in three patients with a loss-of-function mutation in CD-GEFI. In their studies, Canault et al., found that platelets from heterozygous patients, who did not show defects in hemostasis, exhibited a significant adhesive defect under flow conditions[14]. Thus, the various studies in knockout mice and patients suggest partial inhibition of CD-GEFI as a powerful yet safe strategy to prevent thrombosis. In the present study, we describe a hypomorphic mouse strain expressing low levels of human CD-GEFI (*Cdg1*^{low}) instead of the endogenous mouse CD-GEFI. Platelets from *Cdg1*^{low} mice showed decreased platelet activation when compared to WT controls. Importantly, however, their integrin activation response was significantly stronger than that of *Cdg1*$^{-/-}$ mice. Consistent with the *in vitro* integrin activation phenotype, *Cdg1*^{low} mice exhibited only a mild defect in primary hemostasis while they were strongly protected from experimental thrombosis.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement

RESULTS

Generation of hypomorphic *Cdg1* mutant mice

The platelet and hemostasis phenotype of *Cdg1*$^{-/-}$ mice has been characterized extensively. To investigate the regulation of CD-GEFI function in platelets by genetic means, we aimed to establish a cDNA knock-in model where *WT* or mutant human *Cdg1* cDNA is knocked into the murine *Cdg1* locus (Figure 1). We selected to express human CD-GEFI variants in mice, as (1) there is 96% and 100% amino acid sequence identity between human and murine CD-GEFI and RAP1B, respectively, and (2) this approach would allow us to evaluate the ability of human CD-GEFI variants to support platelet activation and plug formation in mice. We noticed, however, that the cDNA knock-in of human *Cdg1* led to markedly reduced expression of CD-GEFI protein in platelets isolated from these mice (~90% reduction compared to controls) (Figure 2A), while the expression of RASA3, RAP1, or β-ACTIN was not affected. Just like *Cdg1*$^{-/-}$ mice[4], these *Cdg1*^{low} mice exhibited a small but significant increase in circulating neutrophils, but they did not exhibit changes in the peripheral platelet count or the platelet size (Table 1). However, compared to WT controls, platelets from *Cdg1*^{low} mice showed marked defects in RAP1 activation in response to agonist stimulation (Figure 2B). Thus, cDNA knock-in of human *Cdg1* strongly reduced platelet CD-GEFI expression and thus led to the generation of hypomorphic *Cdg1* mutant mice.

Impaired integrin activation and aggregation of *Cdg1^{low}* platelets

Consistent with the defect in RAP1 activation, platelets from *Cdg1^{low}* mice were markedly impaired in their ability to activate αIIbβ3 integrin (Figure 3A, C, E) and to aggregate when stimulated with various doses of Par4 activating peptide (Par4p, Figure 4 A, B), the collagen mimetic convulxin (Cvx, Figure 4 C, D), or ADP (Figure 4, E, F). *Cdg1^{low}* platelets also showed a significant defect in α-granule release in response to activation with Par4p (Figure 3B) or Cvx (Figure 3D). Importantly, however, integrin activation, granule release and aggregation were significantly increased in *Cdg1^{low}* platelets when compared to platelets from *Cdg1$^{-/-}$* mice. Thus, expression of low levels of human CD-GEFI partially rescued integrin activation in platelets from mice lacking endogenous CD-GEFI. We next investigated whether this increase in integrin function translated to improved platelet adhesion to collagen under flow conditions. As expected, platelets in *Cdg1^{low}* blood exhibited markedly impaired adhesive function when compared to *WT* controls (Figure 5). However, both platelet accumulation and platelet coverage of the collagen surface were significantly higher in *Cdg1^{low}* blood when compared to the *Cdg1$^{-/-}$* sample, both under low (Figures 5 A,B,C) and high (Figures 5 D,E,F) shear stress conditions. As shown in Figures 5C and F, *Cdg1^{low}* platelets only formed three-dimensional thrombi when perfused over collagen at low shear conditions, confirming our previous observations that CD-GEFI is particularly important for platelet adhesion under arterial shear stress conditions[12].

Cdg1^{low} mice show mild defect in hemostasis

A loss-of-function mutation or genetic knockout of *Cdg1* leads to moderate to severe bleeding upon challenge in humans[14] and mice[4, 12], respectively. It is important to note, however, that, unlike mice deficient in the integrin adapters talin-1 or kindlin-3[15, 16], complete deficiency in CD-GEFI does not lead to perinatal bleeding and increased mortality. Consistent with these findings, *Cdg1^{low}* mice showed no signs of perinatal bleeding or reduced viability (not shown). To evaluate hemostasis, we subjected *Cdg1^{low}* mice to a model of precise laser-induced injury to the saphenous vein[17] as well as the more widely used tail bleeding time assay. Injuries in the saphenous vein bleeding model are small (<100μm in diameter) and thus can be imaged and analyzed by intravital microscopy for platelet adhesion (Figures 6A,B) and time to hemostatic plug formation (Figures 6A,C) (also see supplemental videos 1,2, and 3). Compared to WT controls, both platelet adhesion and hemostasis were markedly impaired in *Cdg1$^{-/-}$* mice. A significant reduction in platelet adhesion and a delay in hemostatic plug formation were also observed in *Cdg1^{low}* mice. However, while *Cdg1$^{-/-}$* mice bled for the entire observation period (300 sec), hemostasis was achieved within about 80 seconds in *Cdg1^{low}* mice. The delay in hemostatic plug formation observed in *Cdg1^{low}* mice correlated well with a delay in platelet adhesion at the site of laser injury. Consistent with the findings in this small injury hemostasis model, we also observed markedly decreased blood loss from severed tails of *Cdg1^{low}* mice when compared to *Cdg1$^{-/-}$* mice (Figure 6D). In fact, blood loss in *Cdg1^{low}* mice was not significantly higher than that in *WT* control mice, even though we observed continuous "oozing" of very small amounts of blood from the transected tails of about 60% of *Cdg1^{low}* mice (Figure 6E). Together, these studies suggest that expression of ~10% of CD-GEFI in platelets is sufficient to maintain hemostasis in mice.

Cdg1^{low} mice are strongly protected from experimental thrombosis

Mice lacking CD-GEFI are strongly protected from arterial thrombosis and ITT[12, 13]. Given their minor hemostatic defect, we were curious to see whether *Cdg1^{low}* mice were still protected from experimental thrombosis. Similar to *Cdg1$^{-/-}$* mice, *Cdg1^{low}* mice did not form occlusive thrombi in a model of $FeCl_3$-induced injury to the carotid artery (Figure 7A,B). In this model, vascular occlusion is defined as a reduction of blood flow by >75%, caused by the formation of a large thrombus. Since thrombus formation was not directly visualized in this model, we were not able to compare platelet adhesion to sites of $FeCl_3$ injury between *WT, Cdg1$^{-/-}$* and *Cdg1^{low}* mice. We did, however, visualize platelet adhesion in the laser injury hemostasis model (Figure 6A, supplemental videos 1–3). In *WT* mice, hemostatic plug formation was characterized by a first phase of rapid growth that was followed by "pacification" of the thrombus, i.e. the shrinking of the thrombus down to a core region. The observed kinetics of platelet adhesion to sites of laser injury in *WT* mice are consistent with studies by Brass and colleagues, who demonstrated that thrombus pacification is due to the reversible binding of weakly activated platelets in the shell region of the thrombus[18]. Interestingly, thrombi in *Cdg1^{low}* mice were markedly smaller and did not seem to contain a shell region. Thus, it is likely that *Cdg1^{low}* mice formed very small platelet thrombi at sites of $FeCl_3$ injury, but that these thrombi cannot grow big enough to impede the blood flow in this high shear stress environment.

We also subjected *Cdg1^{low}* mice to a recently established model of ITT[13]. In this model, platelet activation and pulmonary embolism is triggered via FcγRIIA, a receptor that is expressed on human but not murine platelets. To circumvent this limitation, these studies were performed with transgenic mice expressing human FcγRIIA[19]. *Fcr2a-tg* and *Cdg1^{low}Fcr2a-tg* mice were injected with an antibody against GPIX, a subunit of the platelet Von Willebrand receptor complex. As shown recently, anti-GPIX antibody treatment of mice leads to platelet activation and pulmonary embolism in an FcγRIIA-dependent fashion[13]. Consistent with previous results, injection of anti-GPIX antibody into *Fcr2a-tg* mice led to a >80% decrease in the peripheral platelet count (Figure 7C) and the formation of large pulmonary emboli (Figures 7D,E). In contrast, the peripheral platelet count in *Cdg1^{low}Fcr2a-tg* mice injected with anti-GPIX antibody dropped by only ~30% and platelet accumulation in the lungs was dramatically decreased compared to *Fcr2a-tg* mice.

DISCUSSION

Current strategies to prevent excessive platelet activation and arterial thrombosis include inhibitors of the main platelet integrin, αIIbβ3, and drugs targeting feedback signaling by ADP and TxA_2 (P2Y12 antagonists and aspirin; also known as dual anti-platelet therapy – DAPT[20, 21]). αIIbβ3 antagonists provide very powerful protection from thrombosis but also markedly increase the patients' risk for pathological bleeding. In comparison, DAPT provides weaker protection from thrombosis but is safer with regard to unwanted bleeding. To provide a significant improvement over existing therapies, the next generation of anti-platelet drugs should be as effective as αIIbβ3 inhibitors in preventing thrombosis, but with a safety (bleeding) profile similar or better than that of DAPT.

Here we report that mice expressing small amounts of CD-GEFI are strongly protected from arterial and immune complex-mediated thrombosis. Importantly, as compared to *Cdg1*$^{-/-}$ mice, this protection in *Cdg1*^{low} mice does not come at the expense of a deficient hemostatic response. Thus, small amounts of functional CD-GEFI are sufficient to preserve most of the hemostatic function of platelets, but not sufficient for the formation of pathological thrombi. This phenotype is similar to that of mice with impaired, but not abolished, expression/function of the integrin adapter proteins, TALIN-1[22] or KINDLIN-3[23]. In all cases αIIbβ3-mediated platelet aggregation is delayed, allowing for the formation of small hemostatic plugs. If confirmed in humans, these findings would suggest TALIN-1, KINDLIN-3, and CD-GEFI as promising new targets for anti-platelet therapy. Compared to TALIN and KINDLIN, however, CD-GEFI may be a preferred target as (1) its expression is largely confined to platelets and neutrophils, and (2) mice deficient in talin-1[15] or kindlin-3[16], but not *Cdg1*$^{-/-}$ mice exhibit spontaneous bleeding and high perinatal mortality. Interestingly, impaired platelet function and bleeding upon challenge are the main phenotypes of dogs[24] and humans[14, 25] lacking functional CD-GEFI, suggesting that neutrophil function is less dependent on the CD-GEFI/RAP1 signaling pathway. Our work in *Cdg1*$^{-/-}$ mice identified significant defects in neutrophil integrin activation and adhesion[26], but these defects were mild when compared to those observed in knockout platelets or those in neutrophils lacking kindlin-3[27]. As neutrophils are known modulators of experimental thrombosis[28], however, studies in mice lacking CD-GEFI in platelets or neutrophils only will be required to determine whether impaired neutrophil function contributes to the anti-thrombotic phenotype observed in CD-GEFI mutant mice.

Based on our studies, we propose that targeting CD-GEFI could provide certain advantages over existing antiplatelet therapies. While P2Y12 inhibitors affect the sustained activation of RAP1 and thus protect from thrombosis by destabilizing existing thrombi[12], lack of functional CD-GEFI delays platelet activation and impairs thrombus formation in mice[4, 12, 29] and humans[14, 25]. CD-GEFI also plays a critical role for ITAM-dependent platelet activation[29], and both knockout[12, 13, 30] and *Cdg1*^{low} mice are strongly protected from arterial and IC-mediated thrombosis. Thus, inhibitors of CD-GEFI are expected to provide significantly better protection from thrombosis than drugs targeting P2Y12. Obviously, CD-GEFI inhibitors would have to be carefully monitored in patients, as complete lack of function in this protein is associated with a marked bleeding risk in humans and mice[8, 9, 12, 14, 25]. Alternatively, smarter strategies to inhibit CD-GEFI function could be developed. For example, our recent work demonstrated that deletion of the C1 regulatory domain in CD-GEFI leads to an ~70% reduction in GEF activity[12]. If we succeed in identifying how the C1 domain contributes to CD-GEFI function, inhibitors could be developed that specifically target this regulatory domain. Such inhibitors would not need to be titrated in patients as complete inhibition of CD-GEFI could not be achieved with such an approach. Lastly, inhibitors to CD-GEFI could be used as a safer alternative to αIIbβ3 inhibitors currently used in high-risk patients. The studies reported here combined with our previous work suggest that the antithrombotic effect of a putative CD-GEFI inhibitor would be comparable to that of αIIbβ3 inhibitors – but at a lower risk for bleeding.

In summary, we provide evidence that low-level expression of CD-GEFI leads to protection from thrombosis, but not to marked bleeding, in mice. Based on these findings, we propose

that specific targeting of CD-GEFI would provide a significant improvement over clinically used anti-platelet therapies, such as αIIbβ3 inhibitors and DAPT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

Cdg1	CalDAG-GEFI
Cvx	Convulxin
Par4p	Par4 activating peptide
DAPT	Dual antiplatelet therapy
Fcr2a-tg	FcγRIIa transgenic mice
ITT	Immune-mediated thrombocytopenia and thrombosis

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HIGHLIGHTS

- Low level expression of CalDAG-GEFI is sufficient for the hemostatic function of platelets
- Mice expressing low levels of CalDAG-GEFI are fully protected from experimental thrombosis
- Partial inhibition of CalDAG-GEFI may provide a powerful yet safe approach to prevent thrombosis

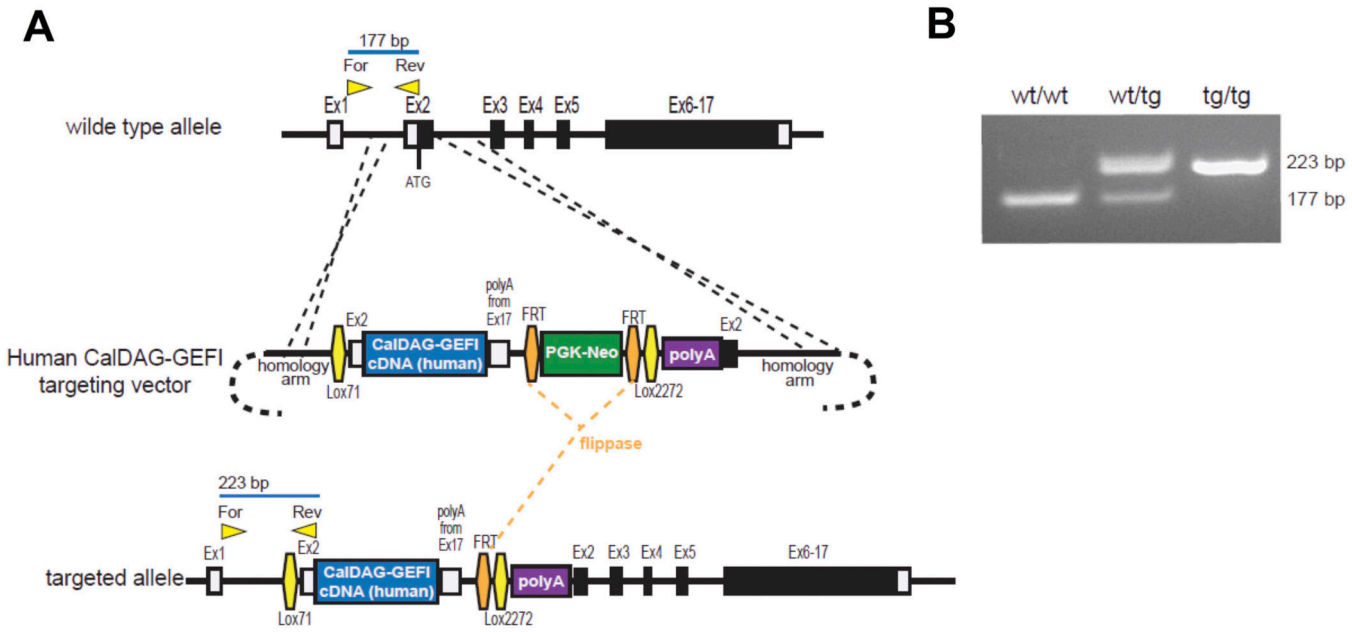

Figure 1. Generation of a CalDAG-GEFI humanized mouse model

(**A**) Schematic of human CalDAG-GEFI knock-in strategy. A gene targeting vector was constructed to insert the human CalDAG-GEFI cDNA at the start codon of the mouse CalDAG-GEFI locus with a copy of the mouse 3'UTR/polyadenylation sequence placed immediately downstream of the human cDNA. A FRT-flanked PGK-Neo resistance cassette was placed downstream of the expression cassette, followed by an additional polyadenylation signal. Lox71 and Lox2272 sites were inserted flanking the cDNA cassette and selectable marker to allow replacement of the cassette by recombinase-mediated cassette exchange strategy. Positions of forward and reverse genotyping primers are indicated along with the size of PCR products to be obtained from the wild-type and knock-in alleles. (**B**) PCR genotyping of animals. Primers shown in panel A were used to amplify DNA from wild-type (wt/wt), heterozygous knock-in (wt/tg) and homozygous knock-in (tg/tg) mice.

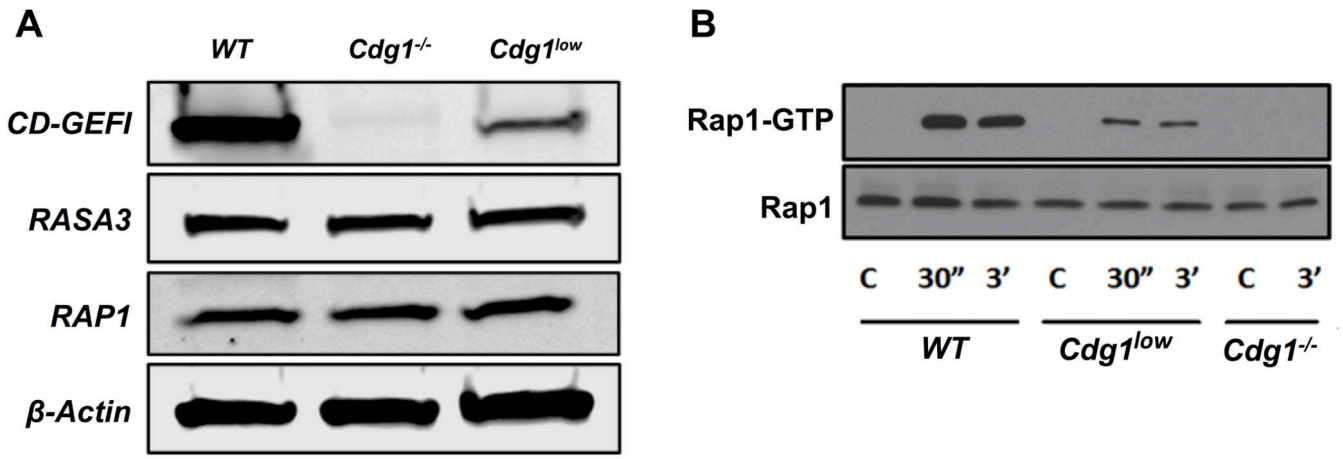

Figure 2. Markedly reduced expression of CD-GEFI in hypomorphic mice (*Cdg1*^{low})

A) Representative Western blots for CD-GEFI, RASA3, RAP1, and β-ACTIN in platelet lysates from *WT*, *Cdg1*$^{-/-}$, and *Cdg1*^{low} mice. **B**) RAP1-GTP levels (top panel) in *WT*, *Cdg1*$^{-/-}$, and *Cdg1*^{low} platelets left unstimulated (C) or activated with PAR4 peptide for 30" or 3'. Total RAP1 is provided as a loading control (bottom panel). Results are representative of 3 independent experiments.

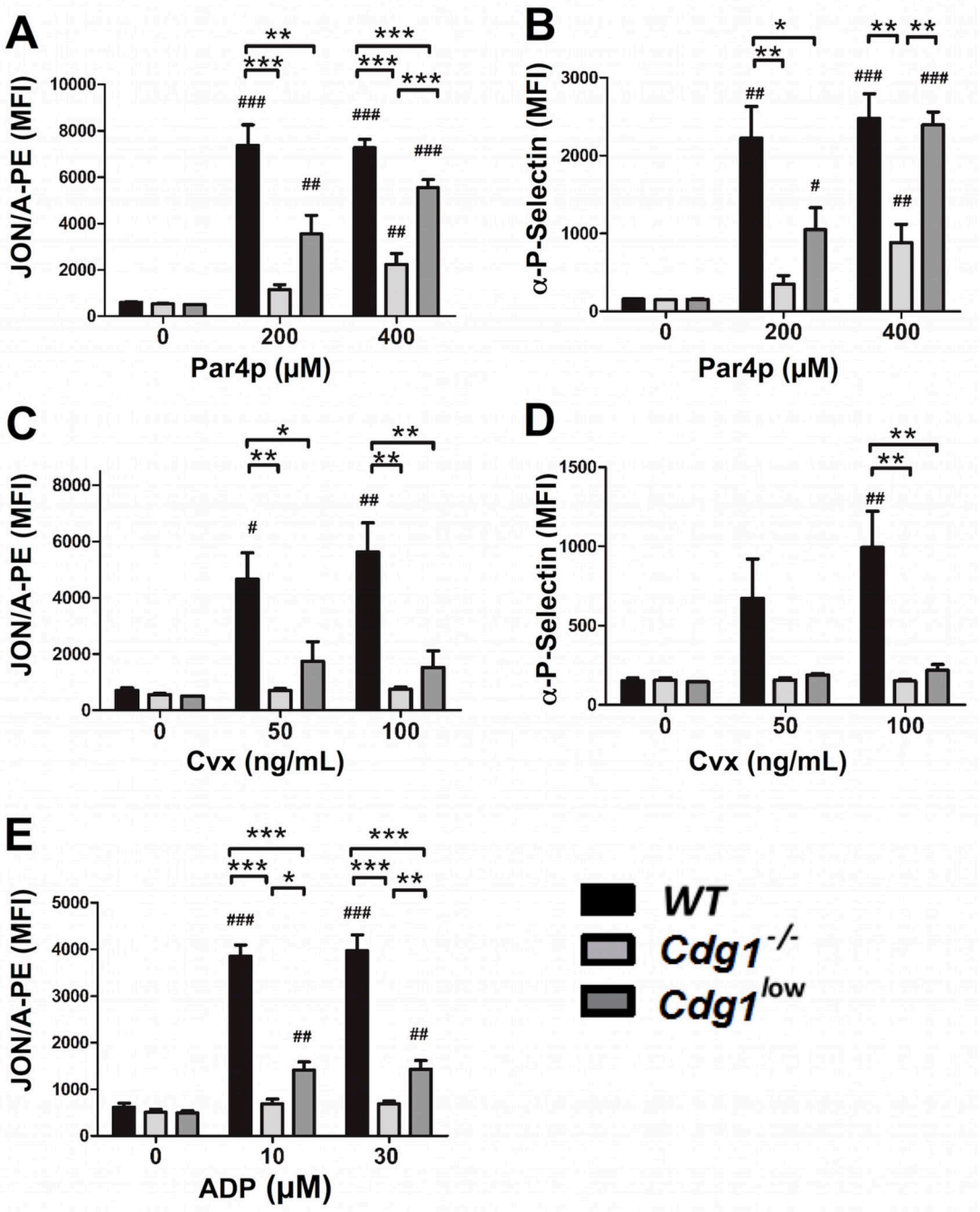

Figure 3. Improved integrin activation in *Cdg1*^{low} compared to *Cdg1*$^{-/-}$ platelets

(**A-E**) αIIbβ3 integrin activation and alpha granule release. JON/A-PE (A,C,E) and anti-P-selectin Alexa Fluor 488 (B,D) binding to platelets from the indicated mice, activated with various concentrations of PAR4p (A,B), convulxin (Cvx; C,D), or ADP (E). Data are shown as mean fluorescence intensity (MFI) ± SEM; n=4–6, *p<0.05, **p<0.01, ***p<0.001; # p<0.05, # #p<0.01, ### p<0.001 relative to resting controls, analyzed by two-way ANOVA with a Bonferonni post test.

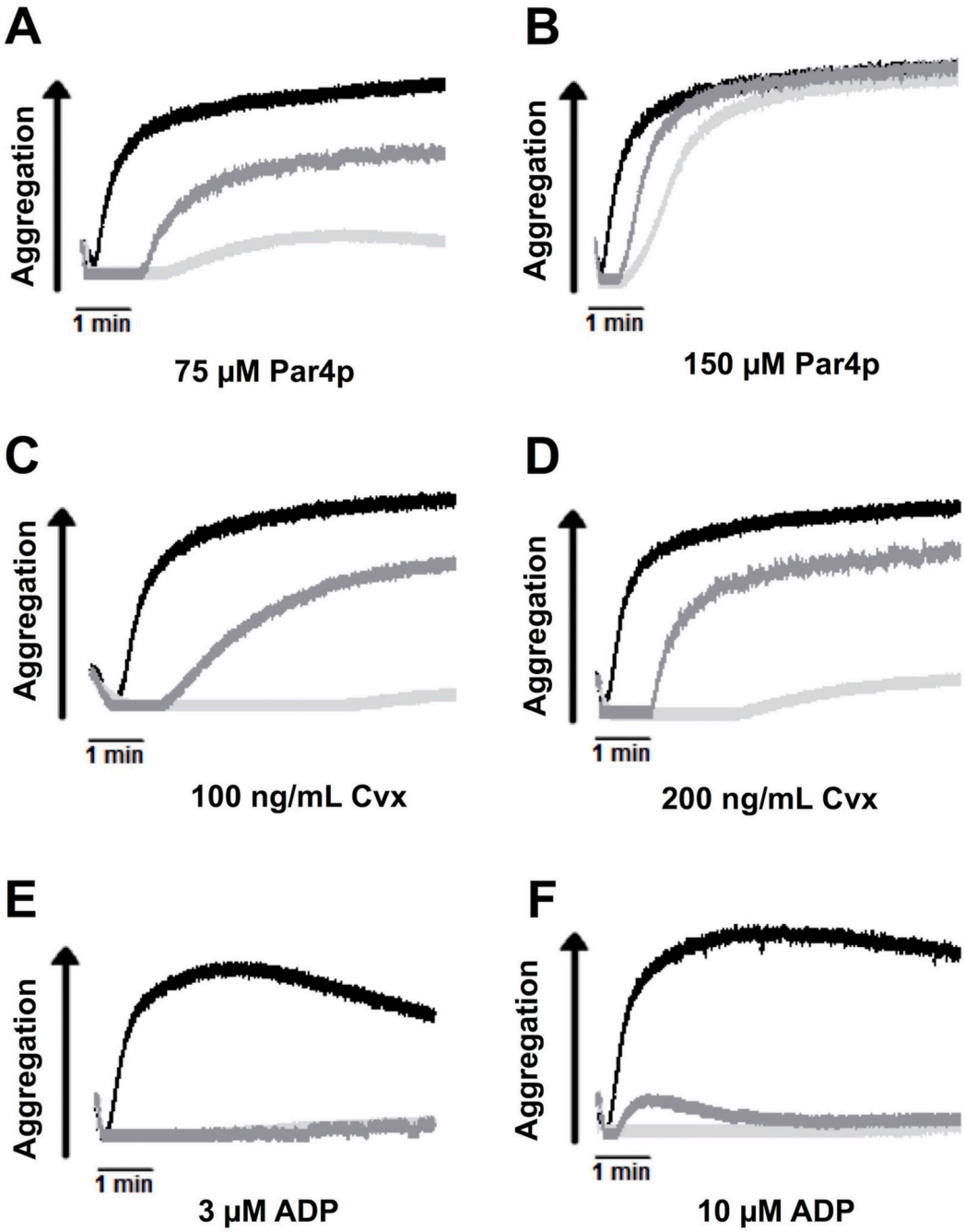

Figure 4. Intermediate aggregation response of *Cdg1^{low}* platelets compared to controls

(**A-F**) Representative aggregometry traces for *WT (black line)*, *Cdg1$^{-/-}$ (light grey)*, and *Cdg1^{low}* (dark grey) platelets activated with the indicated agonists. Aggregometric responses to 75 (A) and 150 (B) μM Par4p, 100 (C) and 200 (D) ng/mL convulxin, and 3 (E) and 10 (F) μM ADP.

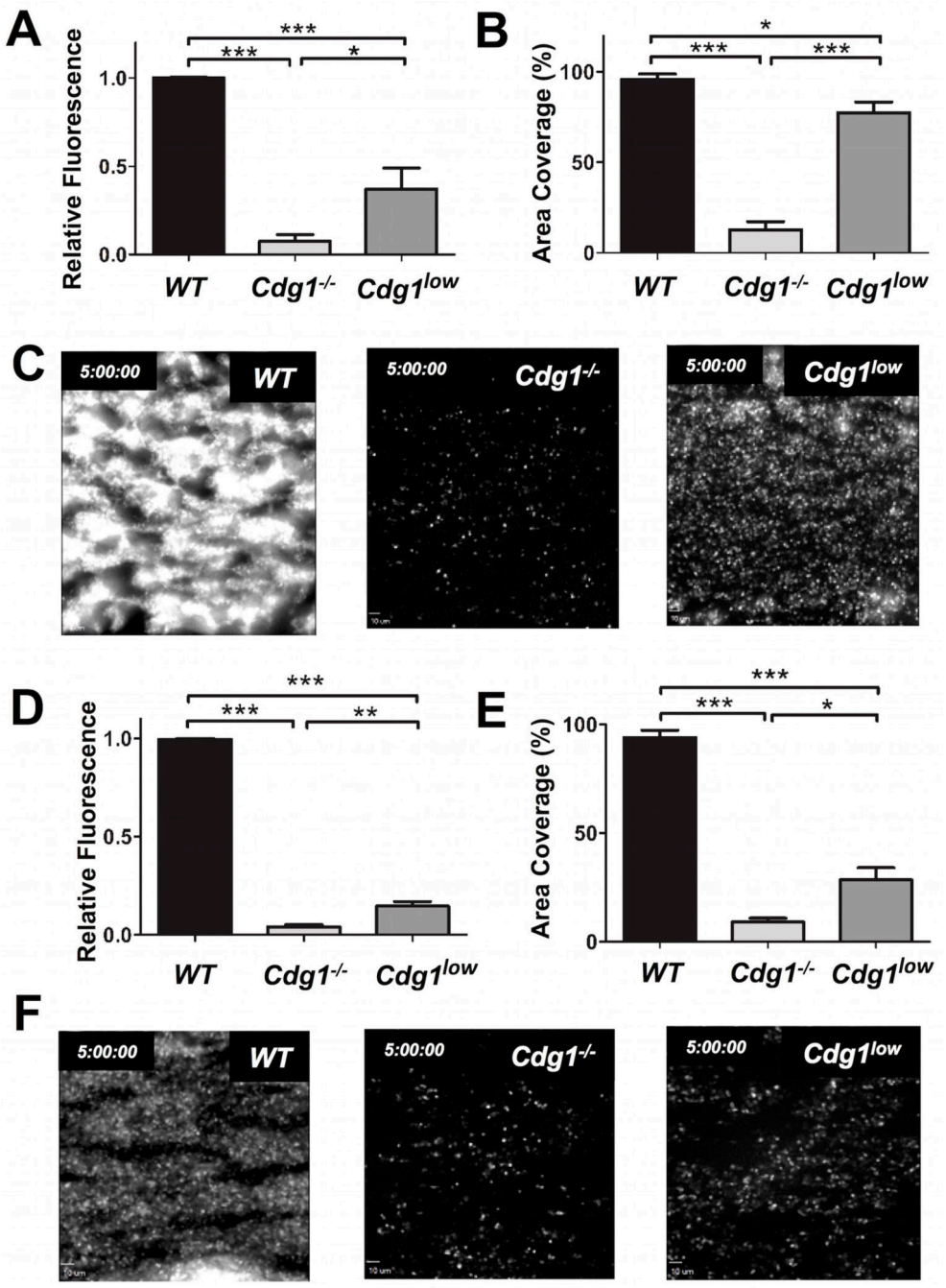

Figure 5. Improved platelet adhesion to collagen of *Cdg1^{low}* compared to *Cdg1$^{-/-}$* platelets

Whole blood from *WT* (black bars), *Cdg1^{low}* (dark grey), and *Cdg1$^{-/-}$* mice (light grey bars) was perfused for 5 minutes over fibrillar collagen (200 μg/mL) at venous (400 s^{-1}, **A-C**) or arterial (1600 s^{-1}, **D-F**) shear rates. Platelets were labeled with Alexa Fluor 488-labeled antibodies to GPIX before perfusion. At the end of the perfusion period, fluorescence images (C,F) were taken and the sum intensity (A,D) and the surface area coverage (B,E) were determined. Fluorescence intensities were normalized to the maximum intensity measured in *WT* control samples (Relative Fluorescence). Area coverage represents the area

in the field of view covered by fluorescently labeled platelets. Data are shown as mean ± SEM, (n = 5) *p<0.05, **p<0.01, ***p<0.001. Images taken on a Nikon TE300 equipped with a QImaging Retiga Exi CCD camera, 20x/0.5 magnification, Alexa Fluor 488-labeled GPIX, Slidebook 5.0 Software at room temperature.

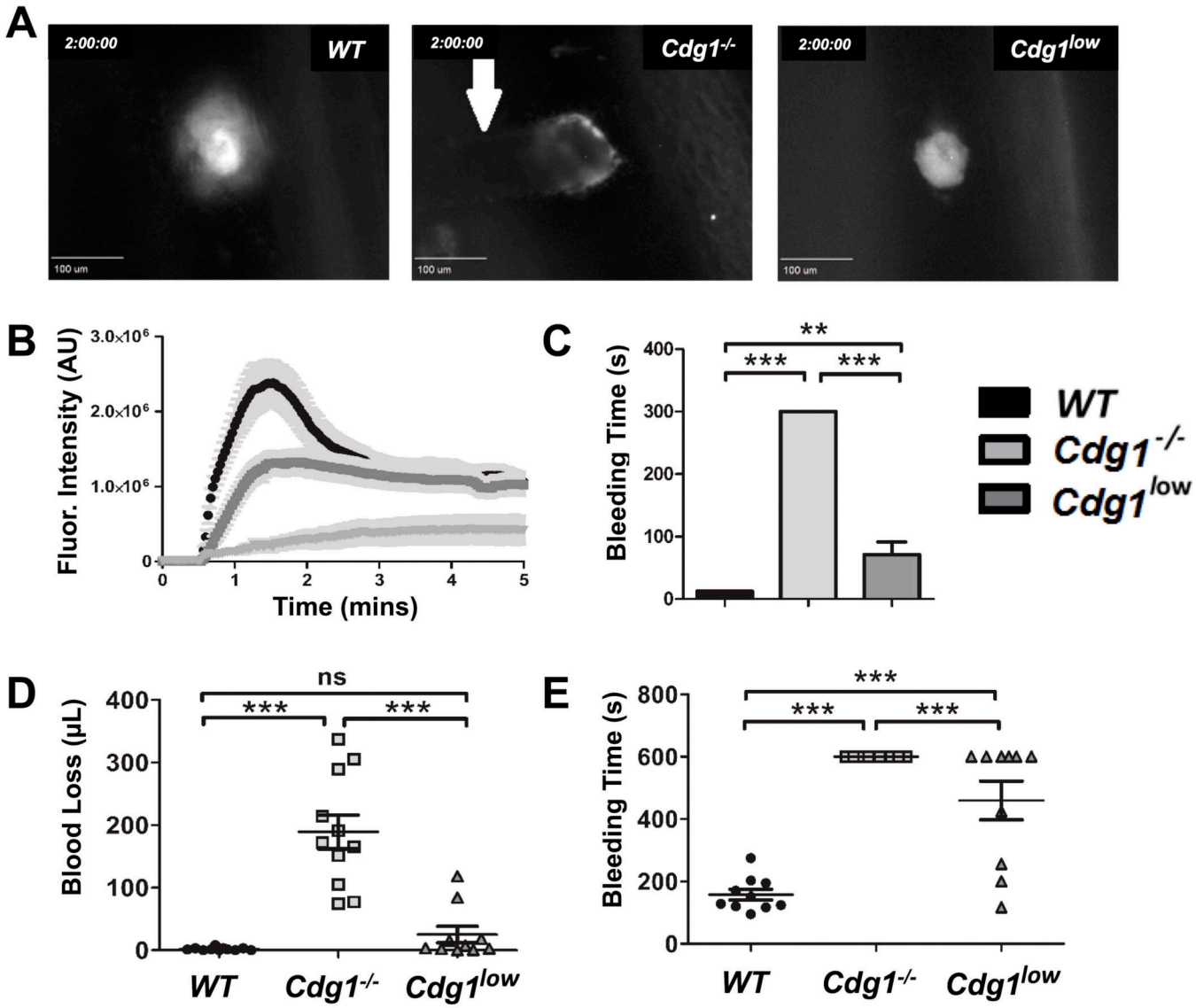

Figure 6. Improved hemostasis in *Cdg1*^{low} compared to *Cdg1*$^{-/-}$ mice

(**A-C**) Intravital microscopy studies to monitor hemostatic plug formation after laser injury to the saphenous vein in WT (black; also see Supplemental Video 1), *Cdg1*$^{-/-}$ (light grey; also see Supplemental Video 2), and *Cdg1*^{low} mice (dark grey; also see Supplemental Video 3). Prior to laser injury, animals were injected with Alexa Fluor 488–labeled antibodies to GPIX. (A) Representative images taken 90 seconds after laser injury. Arrow highlights blood loss at the site of injury in *Cdg1*$^{-/-}$ mice. Scale bar: 100 µm. (B) Sum fluorescence intensity ± SEM recorded at the site of injury over time in the indicated mice (n = 5–11). (C) Time to stable occlusion (no leakage of blood for more than 60 seconds) of the vascular lesion in the indicated mice. Recordings were stopped 300 seconds after laser injury. (**D,E**) Blood loss (D) and bleeding times (E) in *WT* (black circle), *Cdg1*$^{-/-}$ (light gray square), and *Cdg1*^{low} (dark gray triangle) mice after tail transection. ** P<0.01 ***P < 0.001, Chi-square test was performed between groups (n= 10 (WT), 10 (*Cdg1*$^{-/-}$), 10 (*Cdg1*^{low})). Images were taken on

a Zeiss Examiner Z1 equipped with a Hamamatsu C9300 camera, 20x/1 magnification, Alexa Fluor 488-labeled GPIX, Slidebook 5.0 Software at room temperature.

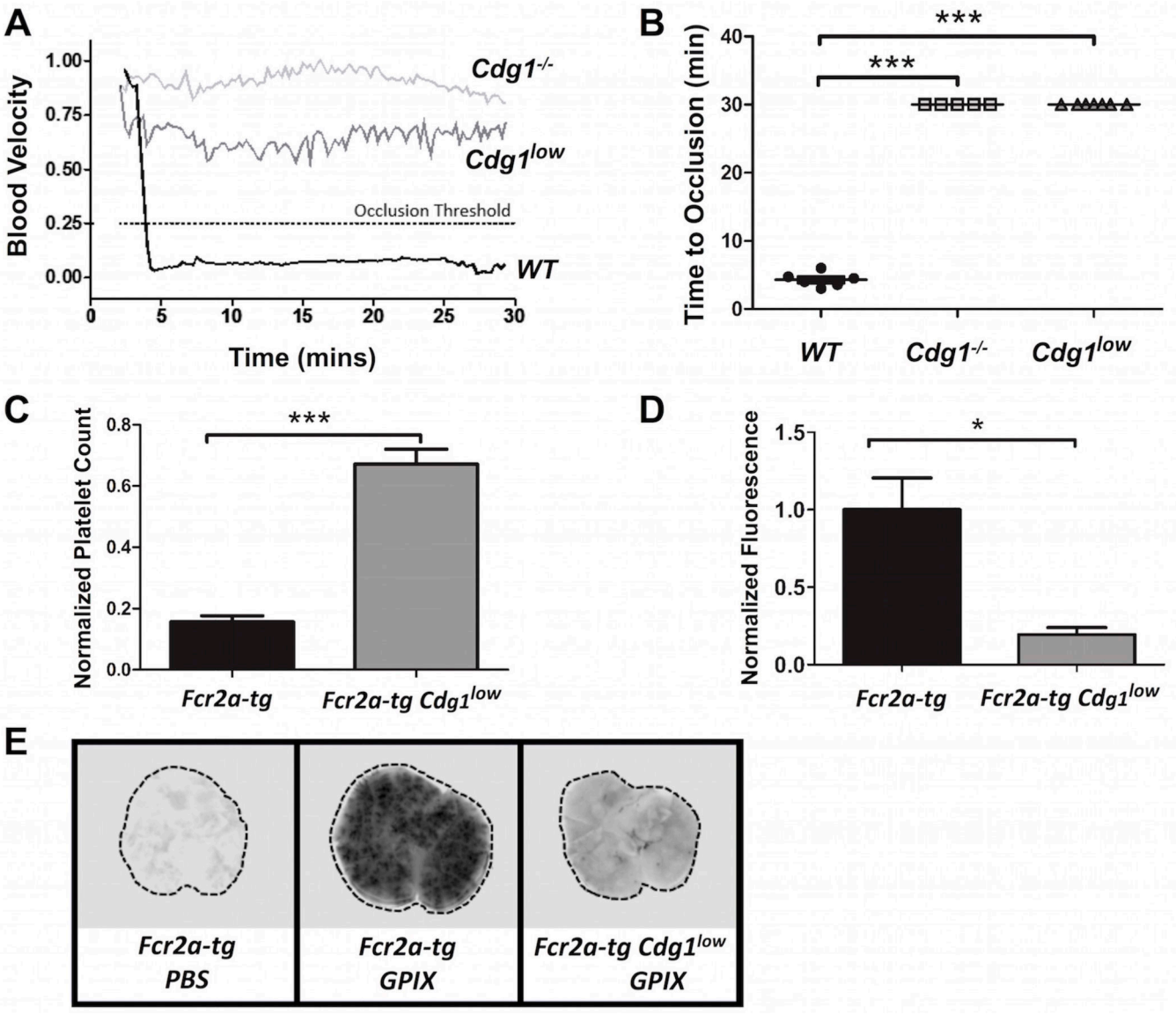

Figure 7. Cdg1^{low} mice are protected from $FeCl_3$- and immune mediated thrombosis

(**A**) Representative blood flow velocity traces recorded after exposure of the carotid artery of a *WT* (black line), *Cdg1*$^{-/-}$ (light grey), or *Cdg1*^{low} (dark grey) mouse to 20% $FeCl_3$ for 1 minute. A decrease in blood flow by > 75% (dotted line) was considered as complete vessel occlusion. (**B**) Time to occlusion recorded in individual *WT* (black circle, n=6), *Cdg1*$^{-/-}$ (light gray square, n=5), and *Cdg1*^{low} mice (dark gray triangle, n=5). (**C**) Platelet counts in whole blood of *Fcr2a-tg* control and *Cdg1*^{low}*Fcr2a-tg* mice 4 hours after administration of 1 μg/g body weight of α-GPIX-IRDye800 antibody. Platelet counts are expressed as percentage of baseline value. (**D,E**) Four hours after antibody infusion, lungs were extracted and scanned on a Li-COR Odyssey at 800 nm. (D) Quantitative analysis of the integrated fluorescence intensity (ImageStudio Lite 4.0). Results are shown as arbitrary fluorescence intensity (a.u.) ± SEM normalized to *Fcr2a-tg* controls; n = 5. *Fcr2a-tg* (black bar), *Cdg1*^{low}*Fcr2a-tg* (gray bar). (E) Representative images. *p<0.05, ***p<0.001.

Table 1

Blood Cell Analysis

	Wild Type	**Cdg1$^{-/-}$**	**Cdg1^{low}**
Platelet Count, K/μL	956 ± 304	906 ± 258	1198 ± 356
Mean Platelet Volume, fL	4.33 ± 0.122	4.189 ± 0.127	4.411 ± 0.136
Neutrophil Count, K/μL	0.70 ± 0.41	2.30 ± 0.757***	1.67 ± 0.7443*
Monocyte Count, K/μL	0.96 ± 0.35	1.584 ± 0.51*	1.231 ± 0.55
Lymphocyte Count, K/μL	7.807 ± 2.92	10.06 ± 1.82	9.033 ± 2.35
Erythrocyte Count, M/μL	12.92 ± 2.26	12.87 ± 1.95	14.43 ± 4.00
Hemoglobin, g/dL	17.89 ± 2.74	19.04 ± 2.79	18.84 ± 3.26

Circulating blood cell analysis. Data are reported as mean ± SD.

* $P < 0.05$,

*** $P < 0.0001$ as compared to wild type control. (n=9)