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Mazharian, Alexandra; Maitre, Blandine; Bornert, Alicia ; Hennequin, Desline; Lourenco-Rodrigues, Marc ; Geer, Mitchell; Smith, Christopher; Heising, Silke; Walter, Michaela; Montel, Florian; Walker, Lucy S K; Salle, Henri De La; Watson, Steve; Gachet, Christian; Senis, Yotis

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American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

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Alexandra Mazharian (Université de Strasbourg, INSERM, EFS Grand-Est, France) Blandine Maître (Université de Strasbourg, INSERM, EFS Grand-Est, BPPS UMR-S1255, FMTS, France) Alicia Bornert (INSERM UMR_S1255, France) Desline Hennequin (EFS Strasbourg, France) Marc Lourenco-Rodrigues (EFS-Grand Est, France) Mitchell Geer (University of Birmingham, United Kingdom) Christopher Smith (University of Birmingham, United Kingdom) Silke Heising (University of Birmingham, United Kingdom) Michaela Walter (Boehringer Ingelheim Pharma GmbH and Company KG, Germany) Florian Montel (Boehringer Ingelheim Pharma GmbH & Co. KG, Germany) Lucy Walker (University College London, United Kingdom) Henri de la Salle (EFS-Alsace, France) Steve Watson (University of Birmingham, United Kingdom) Christian Gachet (EFS, Inserm, France) Yotis Senis (Etablissement Francais du Sang Grand-Est, France)

Abstract:
Mice lacking the immunoreceptor tyrosine-based inhibition motif-containing co-inhibitory receptor G6b-B (*Mpig6b*, *G6b knockout*, *KO*) are born with a complex megakaryocyte (MK)/platelet phenotype characterized by severe macrothrombocytopenia, expansion of the MK population and focal myelofibrosis in the bone marrow and spleen. Platelets are almost completely devoid of the GPVI-FcRγ-chain collagen receptor complex, have reduced collagen integrin α2β1, elevated Syk tyrosine kinase activity, and a subset have increased surface immunoglobulins. A strikingly similar phenotype was recently reported in patients with null and loss-of-function mutations in *MPIG6B*. To better understand the cause and treatment of this pathology, we used pharmacological- and genetic-based approaches to rescue platelet counts and function in *G6b KO* mice. Intravenous-immunoglobulin resulted in a transient partial recovery of platelet counts, whereas immune deficiency had no bearing on platelet count or receptor expression in *G6b KO* mice. Syk loss-of-function (R41A) rescued macrothrombocytopenia, GPVI and α2β1 expression in *G6b KO* mice, whereas treatement with the Syk kinase inhibitor BI1002494 partially rescued platelet counts, but had no effect on GPVI and α2β1 expression, or bleeding. Src family kinase inhibitor dasatinib had no benefit in *G6b KO* mice. In contrast, treatment with the thrombopoietin mimetic romiplostim rescued thrombocytopenia, GPVI expression, and platelet reactivity to collagen, suggesting this may be a promising therapeutic for patients lacking functional G6b-B. Intriguingly, GPVI and α2β1 expression are significantly downregulated in romiplostim-treated *wild-type* mice, whereas GPVI was upregulated in romiplostim-treated *G6b* KO mice, suggesting a cell intrinsic feedback mechanism that auto-regulates platelet reactivity, depending physiological needs.

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Alexandra Mazharian[1,2], Blandine Maître[1], Alicia Bornert[1], Desline Hennequin[1], Marc Lourenco-Rodrigues[1], Mitchell J. Geer[2,3], Christopher W. Smith[2], Silke Heising[2], Michaela Walter[4], Florian Montel[4], Lucy S. K. Walker[5], Henri de la Salle[1], Steve P. Watson[2], Christian Gachet[1] and Yotis A. Senis[1,2]

[1]Université de Strasbourg, Institut National de la Santé et de la Recherche Médicale (INSERM), Etablissement Français du Sang (EFS) Grand-Est, Unité Mixte de Recherche-S 1255, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg, France

[2]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom

[3]Laura and Isaac Perlmutter Cancer Center, New York University School of Medicine, NYU Langone Health, New York, New York

[4] Boehringer Ingelheim Pharma GmbH and Company KG, Ingelheim, Germany

[5] Institute of Immunity and Transplantation, Division of Infection and Immunity, University College London, Royal Free Campus, London, United Kingdom

For original data, please contact the corresponding author: Alexandra.Mazharian@efs.sante.fr.

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Corresponding author: Alexandra Mazharian, UMR-S 1255 INSERM, Université de Strasbourg, EFS Grand-Est, 10 rue Spielmann, F-67065 Strasbourg Cedex, France; email: alexandra.mazharian@efs.sante.fr

Abstract

Mice lacking the immunoreceptor tyrosine-based inhibition motif-containing co-inhibitory receptor G6b-B (*Mpig6b*, *G6b knockout*, *KO*) are born with a complex megakaryocyte (MK)/platelet phenotype characterized by severe macrothrombocytopenia, expansion of the MK population and focal myelofibrosis in the bone marrow and spleen. Platelets are almost completely devoid of the GPVI-FcRγ-chain collagen receptor complex, have reduced collagen integrin α2β1, elevated Syk tyrosine kinase activity, and a subset have increased surface immunoglobulins. A similar phenotype was recently reported in patients with null and loss-of-function mutations in *MPIG6B*. To better understand the cause and treatment of this pathology, we used pharmacological- and genetic-based approaches to rescue platelet counts and function in *G6b KO* mice. Intravenous-immunoglobulin resulted in a transient partial recovery of platelet counts, whereas immune deficiency had no bearing on platelet count or receptor expression in *G6b KO* mice. Syk loss-of-function (R41A) rescued macrothrombocytopenia, GPVI and α2β1 expression in *G6b KO* mice, whereas treatment with the Syk kinase inhibitor BI1002494 partially rescued platelet counts, but had no effect on GPVI and α2β1 expression, or bleeding. The Src family kinase inhibitor dasatinib had no benefit in *G6b KO* mice. In contrast, treatment with the thrombopoietin mimetic romiplostim rescued thrombocytopenia, GPVI expression, and platelet reactivity to collagen, suggesting this may be a promising therapeutic for patients lacking functional G6b-B. Intriguingly, GPVI and α2β1 expression are significantly downregulated in romiplostim-treated *wild-type* mice, whereas GPVI was upregulated in romiplostim-treated *G6b* KO mice, suggesting a cell intrinsic feedback mechanism that auto-regulates platelet reactivity, depending on physiological needs.

Key Points:

1. Romiplostim rescues thrombocytopenia, GPVI expression and platelet collagen reactivity in *G6b KO* mice.
2. IVIG, Syk and Src tyrosine kinase inhibitors have little or no benefit in rescuing the platelet phenotype of *G6b KO* mice.

Introduction

Platelets are essential for primary hemostasis, maintenance of vascular integrity and the innate immune response.[1-3] They have also been implicated in many pathological conditions, including thrombosis, atherogenesis and cancer metastasis.[4,5] Reduced platelet counts, or thrombocytopenia, are defined by their cause, with the most common forms due to autoantibodies to platelet surface receptors, notably the integrin αIIbβ3 and GPIbα subunit, referred to as immune thrombocytopenia (ITP).[6] Immune complex-mediated platelet activation can also lead to enhanced clearance and thrombocytopenia, as is the case in antiphospholipid syndrome,[7] systemic lupus erythematosus,[8] sepsis,[9] heparin-induced thrombocytopenia (HIT) and vaccine-induced immune thrombotic thrombocytopenia (VITT).[10,11] Congenital thrombocytopenias caused by inherited mutations in genes regulating megakaryopoiesis and thrombopoiesis, including May-Hegglin anomaly, Bernard-Soulier syndrome and Gray platelet syndrome, are less prevalent, but clinically significant.[12] Independent of bleeding complications, presence of thrombocytopenia secondary to other conditions is associated with poor prognosis and survival,[13-16] making effective management essential.

The immunoreceptor tyrosine-based inhibition motif (ITIM)-containing co-inhibitory receptor G6b-B, also referred to as *megakaryocyte (MK)/platelet inhibitor G6b* (*MPIG6b*, or *G6b*), has emerged as a critical regulator of platelet homeostasis.[17] Targeted deletion of *Mpig6b* (*G6b knockout, KO*),[18] or uncoupling G6b-B from Src homology 2 domain-containing tyrosine phosphatases 1 and 2 (Shp1 and Shp2),[19-21] resulted in >75% reduction in platelet count and >35% increase in platelet volume. Thrombocytopenia was primarily due to reduced platelet production, although platelet clearance was also significantly increased in *G6b KO* mice.[18] Marked MK expansion, secondary to elevated plasma thrombopoietin levels is observed in G6b-B-deficient mice, with evidence of MK fragmentation and platelet release in the bone marrow. Focal myelofibrosis around clusters of MKs is a prominent feature, and increased prevalence of osteosclerosis in aged mice, likely driven by elevated MK-derived TGF-β1 release and signaling.[18,21,22] Platelets from

these mice are almost completely devoid of the GPVI-FcR γ-chain collagen receptor complex, have reduced collagen integrin α2β1, elevated Syk tyrosine kinase activity, and a subset have increased surface immunoglobulins.[18] Several patients from unrelated families have been reported with mutations in *MPIG6B* and little or no functional G6b-B.[23-27] Homozygous patients presented with similar symptoms to G6b-B null and loss-of-function mouse models, including severe macrothrombocytopenia, MK expansion and focal myelofibrosis from an early age. Successful treatment of the most severely affected family members required bone marrow transplantation.[24-27]

Overlapping phenotypes of G6b-B-deficient mice and humans demonstrates the importance of this receptor to both species, and provides an animal model to investigate the molecular basis of the disease and efficacy of therapies. Such a study is pertinent with ~~to~~ the emergence of an increasing number of disease-causing mutations in *MPIG6B*. Standard clinical therapies for thrombocytopenia were investigated, including administration of intravenous immunoglobulin (IVIG) and Syk tyrosine kinase inhibition, to block platelet clearance, and the thrombopoietin mimetic romiplostim, to boost MK/platelet production. Whereas IVIG and Syk inhibition had partial therapeutic benefits, romiplostim proved the most efficacious in rescuing platelet count, GPVI expression and platelet reactivity to collagen in *G6b KO* mice. Intriguingly, romiplostim reduced expression of GPVI and the collagen integrin α2β1 in *wild-type* (*WT*) mice, suggesting a cell intrinsic feedback mechanism that autoregulates platelet reactivity depending on physiological needs.

Materials and methods

Mice

G6b KO (*G6b*$^{-/-}$) mice were generated on a C57BL/6 background by Taconic Artemis as previously described.[18] *Recombination-activating gene 1* (*Rag1*) *KO* (B6.129S7-*Rag1*^{tm1Mom}/J) mice were obtained from Jackson Laboratory. Cre-inducible *Syk R41A knock-in* (*KI*) mice (*Syk R41A*$^{fl/fl}$) were generated by Ozgene (Bentley, Australia) and crossed with *Pf4-Cre*$^{+}$ mice as described previously.[28] *G6b KO*;*Syk R41A KI*;*Pf4-Cre*$^{+}$mice and *G6b/Rag1 double KO (DKO)* mice were bred by crossing *Syk R41A KI*;*Pf4-Cre*$^{+}$ and *Rag1 KO* mice, respectively, with *G6b KO* mice. Control mice were pure C57BL/6 (*G6b*$^{+/+}$), referred to as *WT*. All procedures were undertaken with the U.K. Home Office approval in accordance with the Animals Scientific Procedures Act of 1986 and with the French Ministry of Research, in accordance with the guidelines of the Regional Committee for Ethics in Animal Experimentation of Strasbourg (CREMEAS, CEEA-35).

Antibodies and reagents

Romiplostim and IVIG were purchased from the Pharmacy Department of the University Hospital Birmingham, UK. BI1002494 was kindly provided by Boehringer Ingelheim via its open innovation platform opnMe.com.[29] The rat-anti-mouse CLEC2 antibody (17D9) was obtained from Serotech (Oxford, UK). FITC-conjugated GPVI and α2 antibodies were from Emfret Analytics (Eibelstadt, Germany). All other chemicals and antibodies were sourced as previously described.[18]

Blood sample collection and analysis

At indicated time points during experiments, whole blood was collected from tail veins into 5 mM EDTA. At the termination of experiments mice were anesthetized using isoflurane, asphyxiated with CO_2 and bled from the heart or vena cava into 1:10 (v:v) acid-citrate-dextrose. Platelet counts and

volumes were measured using an ABX Pentra 60 Hematology Analyzer (Block Scientific) and an automatic cell counter (Scil Animal Care Company, Altorf, France).

Quantification of autoantibodies

Whole blood from *WT* and *G6b KO* mice was collected weekly from week 4 until week 8. Blood was washed twice to eliminate plasma and circulating Ig. Autoantibodies fixed to platelet surface were then revealed by a double staining with an anti-GPIbα (Ram-1 coupled to Alexa 647) and either with IgM (goat anti-mouse-IgM coupled to Alexa 488) or with IgG (goat anti-mouse-IgG coupled to Alexa 488). Samples were analyzed using a flow cytometer (LSRFortessa™ cell analyzer; BD Biosciences) and data were analyzed using BD FACS Diva software.

Administration of IVIG

Ten-week-old *WT* and *G6b KO* mice received intravenous injections of 2 g/kg of body weight.[30] Blood samples were collected at day 15 from tail veins and at day 20 from the vena cava for analysis of blood parameters.

Administration of romiplostim

Ten-week-old *WT* and *G6b KO* mice received subcutaneous injections of either vehicle (saline) or romiplostim (100 μg/kg of body weight; Amgen) every 3 days, during 3 weeks. Doses and administration schedules were selected according to previous studies in mice.[31] Blood samples were analyzed for platelet count and platelet volume before the onset of the experiment and at days 8 and 15. At day 21, mice were culled and blood was drawn from the vena cava for platelet isolation.

Administration of BI2002494

BI1002494 ($C_{23}H_{24}N_3O_5$)[29] was dissolved in Natrosol 0.5% (Natrosol^{TM} 250 HX Pharma) and applied by oral gavage to ten-week-old *G6b KO* mice at 30 mg/kg of body weight twice a day for 5 days. Blood samples were collected daily from tail veins and from the vena cava at day 5 before analysis of hematology.

Flow cytometry

ACD anticoagulated whole blood were incubated with FITC-conjugated rat-anti-mouse antibodies for GPVI and α2β1 (Emfret, Wurzburg, Germany) and analyzed using an Accuri C6 and Fortessa flow cytometers (BD, Biosciences, Oxford, UK), as previously described.[18]

Analysis of platelet aggregation and secretion of ATP

Washed mouse platelets (2×10^8/ml) were prepared and analyzed for aggregation and ATP secretion with a lumi-aggregometer (Chrono-Log, Havertown, PA, USA), as previously described.[18]

Tail bleeding assay

Tail bleeding experiments were performed on 20–30 g male and female mice, anesthetized with isofluorane and injected with buprenorphine intraperitoneally. The terminal 3 mm of tail was cut using transversally a sharp razor blade and immediately immersed in isotonic saline at 37 °C. Mice were allowed to bleed for a maximum of 30 minutes (min). The bleeding time was defined as the time required for complete arrest of bleeding. If bleeding did not cease after 30 min, the tail was cauterised and 30 min was noted as the bleeding time. The amount of blood lost was estimated by measuring haemoglobin by a colorimetric method.

Statistical analysis

Data presented are means ± standard errors of the mean (SEM). Student's t-test was used to compare sample means and determine statistical significance between pairs of samples. A one-way or two-way analysis of variance (ANOVA) followed by *post-hoc* tests was used to compare differences between multiple observations of more than one variable. $P < 0.05$ was considered statistically significant in all cases.

Results

IVIG partially rescues macrothrombocytopenia in *G6b KO* mice

We previously shown that *G6b KO* mice exhibit thrombocytopenia,[18] which is more severe in females.[22] We demonstrate here that this is the case from 4 weeks old **(Figure 1A)**. Furthermore, previous characterization of the *G6b KO* mouse identified two potential mechanisms for the reduced platelet count, increased platelet clearance and reduced platelet production. Evidence of increased platelet clearance was supported by high levels of IgM and IgG bound to the surface of *G6b KO* platelets.[18] This was further confirmed to be the case from 4 weeks old mice, with *G6b KO* mice exhibiting higher level of IgM and IgG bound to their platelet surface compare to *WT* **(Figure 1B-C)**. Therefore, we first investigated common treatments for ITP ~~immune thrombocytopenia~~. A common first line treatment is administration of IVIG, which acts predominantly by blocking Fc-receptors on phagocytosing cells and reducing platelet clearance. In this study, *WT* and *G6b KO* mice were treated with 2 g/kg IVIG and platelet counts and volumes were monitored at 15 and 20 days post-treatment. We show that administration of IVIG has no effect on platelet count in *WT* mice, but partially rescued the macrothrombocytopenia observed on the *G6b KO* mice 15 days after treatment **(Figure 2A-B)**, with this effect being reduced by day 20. The recovery of platelet counts correlates with a marginal recovery of GPVI and α2β1 expression **(Figure 2C-D)**. Nevertheless, the *G6b KO* platelet count remained significantly lower than levels observed in *WT* mice following treatment with IVIG.

The most recent American Society of Hematology (ASH) guidelines recommend splenectomy,[32] which removes both a site of platelet clearance and a site of anti-platelet antibody production, as a second-line treatment for congenital thrombocytopenia.[33] We therefore genetically ablated immune responses by crossing *G6b KO* mice with immune compromised *Rag1 KO* mice, which lack mature B- and T-cells[34]. *Rag1 KO* mice exhibit normal platelets counts and volumes as shown in **Figure 3A and B**. However, deficiency of the immune response did not rescue the

phenotype in *G6b KO* mice, as *G6b*/*Rag1 DKO* mice exhibit severe macrothrombocytopenia **(Figure 3A-B)** and decreased GPVI and α2β1 expression similar to *G6b KO* mice **(Figure 3C-D)**. Targeting immune-mediated platelet clearance is therefore not effective in ameliorating thrombocytopenia in *G6b KO* mice.

Syk loss-of-function rescues macrothrombocytopenia and GPVI expression

We previously demonstrated that G6b-B inhibits constitutive signaling from GPVI and CLEC-2 in transiently transfected DT40 cells.[35] In addition, we also showed that *G6b KO* platelets have higher basal Syk activity than *WT* platelets,[18] and that G6b-B-Shp2 targets either Syk recruitment or activation downstream of CLEC-2.[20] We therefore investigated whether inhibiting Syk might rescue the thrombocytopenia in *G6b KO* mice. First by using a genetic approach, we utilized the platelet-specific *Syk R41A knock-in* (*KI*) mouse model,[28] which exhibits reduced Syk activity, specifically downstream of ITAM-mediated signaling. *Syk R41A KI* mice were crossed with *G6b KO* mice in order to investigate any potential rescue of the *G6b KO* phenotype. We show that reducing ITAM-mediated Syk activity successfully restores both platelet counts and volumes in *G6b KO* mice to levels observed in *WT* mice **(Figure 4A-B)**, suggesting that G6b-B-mediated Syk inhibition may be essential for normal platelet production. Importantly, GPVI and α2β1 surface expression were also fully restored in *G6b KO* mice on the *Syk R41A* background **(Figure 4C-D)**, implicating increased Syk activity as an underlying cause of downregulation of these receptors in *G6b KO*mice.

Syk inhibition partially restores platelet counts in *G6b KO* mice

To further characterize the contribution of Syk kinase to platelet recovery, we used the selective small-molecule Syk kinase inhibitor BI1002494.[29] Mice were dosed twice a day for five days with 30 mg/kg of BI1002494 per dose. A trend towards increased platelet count was observed in BI1002494-treated male *G6b KO* mice compared with vehicle-treated mice under these conditions,

which was more prominent and significant in female *G6b KO* mice **(Figure 4E)**. Platelet volumes were unaltered under these conditions **(**Figure 4F), as were platelet GPVI and □2□1 surface expression (Figure 4G-H). Platelet aggregation and P-selectin expression were also normal in response to PAR-4 peptide and anti-CLEC-2 antibody in BI1002494-treated mice compared with vehicle-treated mice (**Supplemental Figure 1),** as was blood loss, measured by tail bleeding (Figure 4I-J).

We also sought to assess whether SFK inhibition, which lie upstream of Syk might also be effective to rescue the thrombocytopenia observed in *G6b KO* mice. The effect of *in vivo* administration of dasatinib (5 mg/kg/d for 7 days) on the platelet count was investigated. Treatment with dasatinib caused a reduction in the platelet number in *WT* mice as previously described,[36] however, no significant effect was observed in *G6b KO* mice **(Supplemental Figure 2A-B).**

Collectively, these results demonstrate that the macrothrombocytopenia and aberrant platelet function exhibited by *G6b KO* mice can be only partially rescued by reducing Syk kinase activity, as summarized in **supplemental Table 1**.

Romiplostim rescues thrombocytopenia in G6b-B-deficient mice

Thrombopoietin receptor agonists, including romiplostim and eltrombopag, which drive MK expansion are increasingly utilized in the treatment of thrombocytopenia, whilst splenectomies have declined.[37-39] Romiplostim is a peptibody administered subcutaneously to treat chronic ITP.[37,40] It mimics thrombopoietin binding to its receptor, Mpl, therefore driving differentiation and maturation of MKs.[40] 100 μg/kg of romiplostim was injected into *WT* mice every 3 days, leading to a 4-fold increase in platelet count 8 days after the beginning of the protocol **(Figure 5A)**. Platelet count further increased after 21 days of treatment, reaching a 5-fold increase **(Figure 5A)**. Romiplostim also increased platelet counts in *G6b KO* mice, with a 5-fold increase by day 8, to vehicle treated *WT* levels **(Figure 5A)**, and 8-fold increase by day 21, but significantly below romiplostim-treated *WT*

levels **(Figure 5A)**. A minor increase in the mean platelet volume was observed in romiplostim-treated *WT* mice that was marginally more prominent in female mice, but no further increase was observed with time **(Figure 5B)**. Platelet volume was significantly higher for *G6b KO* mice at baseline, and did not significantly increase in romiplostim-treated *G6b KO* mice **(Figure 5B)**.

Romiplostim increases GPVI expression and collagen reactivity in *G6b KO* mice

We next investigated the effect of romiplostim on expression of the collagen receptors GPVI and α2β1 in *G6b KO* mice. Romiplostim induced a 40% reduction in GPVI expression in *WT* mice, as previously shown[31], and a 25% reduction in α2β1 expression. Unexpectedly, romiplostim induced a 5-fold increase in GPVI expression in *G6b KO* mice **(Figure 5C)**, but had no effect on α2β1 expression **(Figure 5D)**. Despite GPVI levels remaining significantly lower in romiplostim-treated *G6b KO* mice compared with *WT* counterparts, they were much closer than in untreated mice **(Figure 5C)**, and α2β1 levels were the same **(Figure 5D)**.

We also performed platelet function testing *in vitro* by measuring platelet aggregation and ATP secretion, to determine whether romiplostim treatment can rescue other observed platelet defects of the *G6b KO* mice. As previously described,[18] platelets from *G6b KO* mice did not aggregate or secrete ATP in response to the GPVI-specific agonist collagen-related peptide (CRP, 30 and 3 μg/ml), and significantly diminished response to collagen (3 μg/ml) **(Figure 6A-C, Supplemental Figure 3A-C)**, consistent with a reduction in GPVI expression. However, following romiplostim administration, platelets from *G6b KO* mice aggregated normally in response to 30 μg/ml CRP and 3 μg/ml collagen, compared to treated *WT* mice **(Figure 6A, C, Supplemental Figure 3A, C)**. An increased response to 3 μg/ml CRP was also observed in treated versus untreated *G6b KO* mouse platelets, but this was still reduced compared to *WT* **(Figure 6B, Supplemental Figure 3B)**. ATP secretion, a marker of platelet dense-granule secretion, was partially rescued across all concentrations of CRP and collagen tested **(Figure 6A-C, Supplemental Figure 3A-C)**.

In contrast to GPVI stimulation, platelets from *G6b KO* mice exhibit enhanced reactivity to an activating anti-CLEC-2 antibody **(Figure 6D, Supplemental Figure 3D)**. This is due to increased signaling in the absence of inhibition by G6b-B.[18,20] Romiplostim treatment also partially rescued this phenotype, by marginally attenuating the response of *WT* and *G6b KO* platelets to anti-CLEC-2 antibody **(Figure 6D, Supplemental Figure 3D)**. In addition, the reversible aggregation and reduced ATP secretion in response to 0.06 U/ml thrombin in *G6b KO* mice was restored following romiplostim treatment **(Figure 6E)**. These data indicate that romiplostim is effective at rescuing both platelet count and reactivity to various agonists in *G6b* KO mice, as summarized in **supplemental Table 2**.

Discussion

Work presented in this study demonstrates that romiplostim is effective at boosting platelet counts and rescuing platelet function in mice lacking the co-inhibitory receptor G6b-B. In contrast, inhibiting or ablating the immune response to prevent platelet clearance was less efficacious at rescuing the platelet phenotype of G6b-B-deficient mice. Surprisingly, GPVI and α2β1 were downregulated in romiplostim-treated *WT* mice, whereas GPVI was upregulated in *G6b KO* mice following the same treatment, resulting in more comparable platelet responses to collagen and CRP compared with untreated mice **(Figure 7)**. Furthermore, platelets from romiplostim-treated *WT* and *G6b KO* mice responded similarly to anti-CLEC-2 antibody and thrombin compared with untreated mice, suggesting a cell intrinsic feedback mechanism that auto-regulates platelet reactivity depending on physiological needs.

Use of thrombopoietin receptor agonists increases platelet counts and decreases the risk of bleeding in patients with ITP.[41] Two such thrombopoietin receptor agonists, romiplostim and eltrombopag, are approved by the US Food and Drug Administration (FDA) for the treatment of thrombocytopenia.[32] In this study, we show that administration of romiplostim successfully restored platelet counts to physiological levels in *G6b KO* mice, but not to the same extent as in *WT* mice, despite *G6b KO* mice having significantly more MKs to begin with. This correlates with the severe impairment in proplatelet formation and platelet production by G6b-B-deficient MKs, which is the primary cause of thrombocytopenia in these mice.[18] Mean platelet volumes are marginally increased in romiplostim-treated *WT* mice, presumably as more young platelets are released into the circulation, but remained unaltered in *G6b KO* mice, which were already enlarged. In addition, GPVI and α2β1 expression, platelet aggregation and ATP secretion to collagen and thrombin were also rescued. The improved thrombin response was surprising as it acts via G protein-coupled receptors, which are not typically regulated by ITIM-containing receptors. Since *G6b KO* mice have a broad spectrum of morphologically diverse platelets as assessed by electron microscopy,[18] ranging

from platelets with few if any granules, to those with a normal complement of granules, we speculate these findings may be due to skewing of the platelet population towards more biologically active platelets in response to romiplostim. Further preclinical testing is needed to determine effects of G6b-B-deficient MKs, mechanism of action and therapeutic window at which the platelet phenotype and bleeding diathesis are resolved, but myelofibrosis and osteosclerosis associated with G6b-B-deficiency are not exacerbated.[22,41]

One of the most intriguing and unexpected findings of this study is the inverse effects of romiplostim on platelet receptor expression and reactivity in *WT* versus *G6b KO* mice **(Figure 7)**. Our working hypothesis is that MKs and platelets possess cell intrinsic feedback mechanisms that auto-regulate platelet reactivity depending on what is needed for survival. If platelets are biologically normal and counts increase to supra-physiological levels, as was the case in romiplostim-treated *WT* mice, then less active platelets are preferred to guard against unwarranted thrombosis. The opposite is the case in *G6b KO* mice, which require more active platelets to compensate for functional deficits and prevent bleeding. This corresponds with a growing body of evidence that platelet phenotype is dictated by pathological context.[42-46] Underlying mechanisms remain undefined.

Based on our initial characterization of *G6b KO* mice, auto-antibodies were proposed to contribute to the phenotype.[18] Currently, one of the recommended first line treatments advised by the ASH is the administration of IVIG,[32] which blocks Fc receptors on phagocytes, preventing platelet clearance. Administration of IVIG only transiently and partially rescued platelet count, whereas ablation of B- and T-cell populations using *Rag1 KO* mice proved ineffective at increasing *G6b KO* platelet count, demonstrating that anti-platelet auto-antibodies play little or no role in inducing thrombocytopenia in these mice, and arguing against splenectomy as a therapeutic option. Lack of efficacy of inhibiting platelet clearance mechanisms is supported by recent findings from a *G6b-B* loss-of-function mouse model (G6b-B diY/F knock-in),[20] which recapitulate most aspects of

the *G6b KO* phenotype, but present with no clearance defect when platelet kinetics are modelled.[20] In addition to the partial increase in platelet count and reduction in platelet volume with IVIG, the expression of GPVI and the collagen integrin α2β1 were also marginally increased, that may reflect rescue of a subpopulation of platelets targeted for destruction.

Syk inhibitors were recently FDA approved for the treatment of ITP, by inhibiting phagocytosis. As we previously showed that Syk activity is elevated in resting *G6b KO* platelets,[18] we therefore tested the effect of Syk loss-of-function and inhibition on *G6b KO* mice, which may contribute to increased clearance due to pre-activation and GPVI-FcR γ-chain down-regulation, due to tonic signalling. Indeed, Syk loss-of-function rescued platelet count, volume, GPVI and α2β1 expression in *G6b KO* mice. However, the Syk inhibitor BI1002494 had a much milder effect, only partially rescuing platelet count, but neither GPVI and α2β1 expression, nor bleeding. Although this is likely due to the dosing regimen, finding a therapeutic window that corrects platelet count without severely compromising the hemostatic activity of platelets, or the immune response is questionable.

We hypothesize that elevated Syk activity in G6b-B-deficient platelets results in tonic GPVI-FcR γ-chain signalling, triggering negative feedback and downregulation of the entire complex. This would explain why Syk loss-of-function rescues GPVI expression in G6b KO mice. We previously reported increased ADAM-10 on the surface of *G6b KO* mouse platelets,[18] which causes shedding of the GPVI ectodomain. We also described a concomitant reduction in FcR γ-chain expression, which is needed for GPVI expression. Hence, downregulation of GPVI-FcR γ-chain complex in *G6b KO* platelets is multifactorial, the predominant underlying mechanism of which is likely to be context dependent.

Interestingly, expression of the hemITAM-containing receptor CLEC-2, which also signals via Syk, albeit with different kinetics and stoichiometry compared with GPVI-FcR γ-chain,[28,47] is not downregulated in *G6b KO* platelets, despite elevated Syk activity. This may be partially due to the stability of the GPVI-FcR γ-chain-Syk complex compared with the CLEC-2-Syk complex,

comprised of two phosphorylated CLEC-2 receptors and one Syk molecule. The tandem SH2 domains of Syk contribute cooperatively to the high affinity binding to the tandem phospho-tyrosine residues of the FcR γ-chain ITAM, compared with the substantially lower affinity of single Syk SH2 domain binding to monophosphorylated ITAM,[48,49], or one versus two phosphorylated hemITAM CLEC-2 receptors.[47,50] Therefore, the strength and duration of signalling from GPVI-FcR γ-chain might be greater than that from CLEC-2, thus eliciting a more robust negative feedback signal.

In closing, boosting platelet production through the use of thrombopoietin mimetics rescues platelet count and partially rescues platelet reactivity in G6b-B-deficient mice. Further research is required to determine therapeutic window and potential adverse side-effects, particularly pertaining to exacerbation of myelofibrosis and osteosclerosis, as well as investigating cell intrinsic feedback mechanisms that auto-regulate platelet reactivity in response to romiplostim in the presence and absence of G6b-B.

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Authorship Contributions

A.M. Conceptualized, designed and performed experiments, analyzed data, wrote and revised the manuscript.

B.M., A.B., D.H., M.L.R, M.J.G., C.W.S. and S.H. Performed experiments, analyzed data and revised the manuscript.

H.d.l.S. Designed experiments, analyzed data and revised the manuscript.

C.G. Discussed study and revised the manuscript.

L.S.K.W. Provided *Rag1 KO* mice, discussed the study and revised the manuscript.

S.P.W. Provided *Syk R41A KI* mice, discussed the study and revised the manuscript.

Y.A.S. Conceptualized, designed and performed experiments, analyzed data, wrote and revised the manuscript.

ORCID Profiles

A.M. 0000-0002-0204-3325

B.M. 0000-0002-3264-7330

A.B. 0000-0003-4847-6406

M.L.R. 0000-0001-6572-1798

M.J.G. 0000-0003-1457-987X

S.H. 0000-0002-7011-8333

L.S.K.W. 0000-0001-5986-5015

H.d.l.S. 0000-0001-5136-0776

S.P.W. 0000-0002-7846-7423

C.G. 0000-0003-1303-4210

Y.A.S. 0000-0002-0947-9957

Disclosure of Conflicts of Interest

The authors have no competing financial interests to declare.

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Figure legends

Figure 1: G6b-B–deficient platelets have increased surface IgM and IgG abundance

(A) Whole blood platelet counts from young 4 to 8 weeks wild-type (*WT*) and male and female *G6b KO* mice were measured. **(B-C)** Platelets from *WT* and *G6b KO* mice were double-stained for GPIbα and either IgM **(B)** or IgG **(C).** Flow cytometry analysis revealed that *G6b KO* mice exhibit higher level of IgM and IgG bound to their platelet surface compare to *WT*. Data are means ± SEM. n = 12 /condition.

Figure 2: IVIG rescues partially macrothrombocytopenia in G6b-deficient mice.

WT and *G6b KO* mice received 2 g/kg IVIG intravenously once a day for 20 days. **(A)** Platelet counts and **(B)** volumes were measured before (day 0) and after 15 and 20 days of IVIG administration. IVIG partially rescued the macrothrombocytopenia observed in the *G6b KO* mice 15 days after treatment **(A-B)**, with this effect being reduced by day 20. **(C)** GPVI and **(D)** α2β1 platelet surface expression of *WT* and *G6b KO* mice were measured by flow cytometry after 15 days of 2 g/kg IVIG administration. A marginal recovery of GPVI and α2β1 expression was observed in G6b-deficient treated mice. n = 4 mice/genotype/condition, $*P < 0.05$, $***P < 0.001$, two-way ANOVA with Tukey's test, mean ± SEM.

Figure 3: Ablation of the immune system does not rescue the macrothrombocytopenia in G6b-deficient mice. (A) Platelet counts and **(B)** volumes of *WT, G6b KO, Rag1 KO and G6b/Rag1 double-knockout (DKO)* mice were measured, n = 10-26 mice/genotype. *Rag1 KO* mice exhibit normal platelets counts and volumes. However, deficiency of the immune response did not rescue the phenotype in *G6b KO* mice, as *G6b*/*Rag1 DKO* mice exhibit severe macrothrombocytopenia **(A-B). (C)** GPVI and **(D)** α2β1 platelet surface expression of *WT, G6b KO, Rag1 KO and G6b/Rag1*

DKO mice were measured by flow cytometry, n = 8 – 10 mice/genotype. *G6b/Rag1 DKO* mice exhibit decreased GPVI and α2β1 expression similar to *G6b KO* mice. ***$P < 0.001$, one-way ANOVA with Dunnett's test, mean ± SEM.

Figure 4: Reduced Syk activity rescues macrothrombocytopenia and GPVI expression in G6b-deficient mice. **(A)** Platelet counts and **(B)** volumes of *WT, G6b KO, G6b$^{-/-}$R41A$^{+/+}$Cre^{+} and G6b$^{-/-}$R41A$^{fl/fl}$Cre^{+}* mice were measured, n = 9 – 43 mice/genotype. **(C)** GPVI and **(D)** α2β1 platelet surface expression of *WT, G6b KO, G6b$^{-/-}$R41A$^{+/+}$Cre^{+} and G6b$^{-/-}$R41A$^{fl/fl}$Cre^{+}* mice were measured by flow cytometry n = 5 – 18 mice/genotype. ***$P < 0.001$, one-way ANOVA with Dunnett's test, mean ± SEM. **(E-J)** *G6b KO* mice received BI1002494 (30 mg/kg/bid) orally via gavage twice a day for 5 days. **(E)** Platelet counts and **(F)** volumes of *G6b KO* mice were monitored before (day 0) and every day for 5 days, n = 10-15 mice/genotype. ns: non-significant, **$P < 0.01$, ***$P < 0.001$, two-way ANOVA Tukey's test, mean ± SEM. **(G)** GPVI and **(H)** α2β1 platelet surface expression of *WT* and *G6b KO* mice were measured 5 days after BI1002494 administration (30 mg/kg/bid) by flow cytometry, n = 6 - 10 mice/genotype, ns: non-significant. **(I)** Hemostatic response was measured 5 days after BI1002494 administration, in saline tail bleeding assay by an excision of a 3 mm portion of the tail tip followed by immersion of the tail in 0.9% isotonic saline at 37°C. Plotted is the time to complete arrest of bleeding. Each symbol represents 1 animal. **(J)** Blood loss was measured by colorimetric dosage of hemoglobin. Experiment were conducted in a double-blinded manner, n = 7 mice/genotype/condition. One-way ANOVA with Sidak's test, mean ± SD.

Figure 5: Romiplostim rescues thrombocytopenia in G6b-deficient mice.

(A) Platelet counts and **(B)** volumes of *WT* and *G6b KO* mice were measured before (day 0) and after 8, 15, and 21 days of 100 μg/kg of body weight romiplostim administration subcutaneously. Romiplostim increased platelet counts in *G6b KO* mice but did not affect platelet volume. **(C)** GPVI

and **(D)** α2β1 platelet surface expression of *WT* and *G6b KO* mice were measured by flow cytometry before (vehicle) and after 21 days of romiplostim administration. Results are expressed as the mean fluorescent intensity (MFI) ± SEM. In *WT* mice, romiplostim induces a 40% reduction in GPVI expression and 25% reduction in α2β1 expression. However, romiplostim induced a 5-fold increase in GPVI expression in *G6b KO* mice, but had no effect on α2β1 expression. n = 8 mice/condition ns: non-significant, **P* < 0.05, ****P* < 0.001, two-way ANOVA Tukey's test.

Figure 6: Romiplostim rescues platelet reactivity in G6b-deficient mice.

Washed platelet aggregation and ATP secretion of *WT* and *G6b KO* mice before and after 21 days of romiplostim administration were measured by lumi-aggregometry in response to **(A-B)** 30 and 3 μg/ml collagen-related peptide (CRP), **(C)** 3 μg/ml collagen, **(D)** 3 μg/ml CLEC2 Ab and **(E)** 0.06 U/ml thrombin. Following romiplostim administration, platelets from *G6b KO* mice aggregated normally in response to 30 μg/ml CRP and 3 μg/ml collagen, compared to treated *WT* mice **(A, C)** and an increased response to 3 μg/ml CRP was observed in treated versus untreated *G6b KO* mouse platelets (**B**). Romiplostim treatment also partially attenuates the response of *WT* and *G6b KO* platelets to anti-CLEC-2 antibody (**D**) and fully restores reversible aggregation and reduced ATP secretion in response to 0.06 U/ml thrombin in *G6b KO* mice (**E**). Representative traces are shown, n = 5 - 8 mice/genotype/condition.

Figure 7: Effects of romiplostim in wild-type and G6b-B-deficient mice.

Romiplostim is a thrombopoietin receptor agonist that is clinically used to treat chronic immune thrombocytopenia. It mimics thrombopoietin binding to its receptor, Mpl, therefore driving differentiation and maturation of MKs. In this study, we demonstrate that **(A)** treatment of *WT* mice with romiplostim leads to an 8-fold increase in platelet counts to supra-physiological levels. It also reduces GPVI and α2β1 expression in *WT* mice. We hypothesize that romiplostim drives a cell

intrinsic negative feedback pathway that auto-regulates platelet reactivity to collagen depending, suppressing their thrombotic capacity. **(B)** Romiplostim successfully rescues thrombocytopenia of *G6b KO* mice, with an increase of platelet counts to physiological levels. It also partially rescues GPVI expression and platelet reactivity to collagen in *G6b KO* mice, which potentially suggests a loss of negative feedback. Romiplostim proved to be the most effective therapy tested for rescuing the platelet phenotype in *G6b KO* mice.

Figure 1

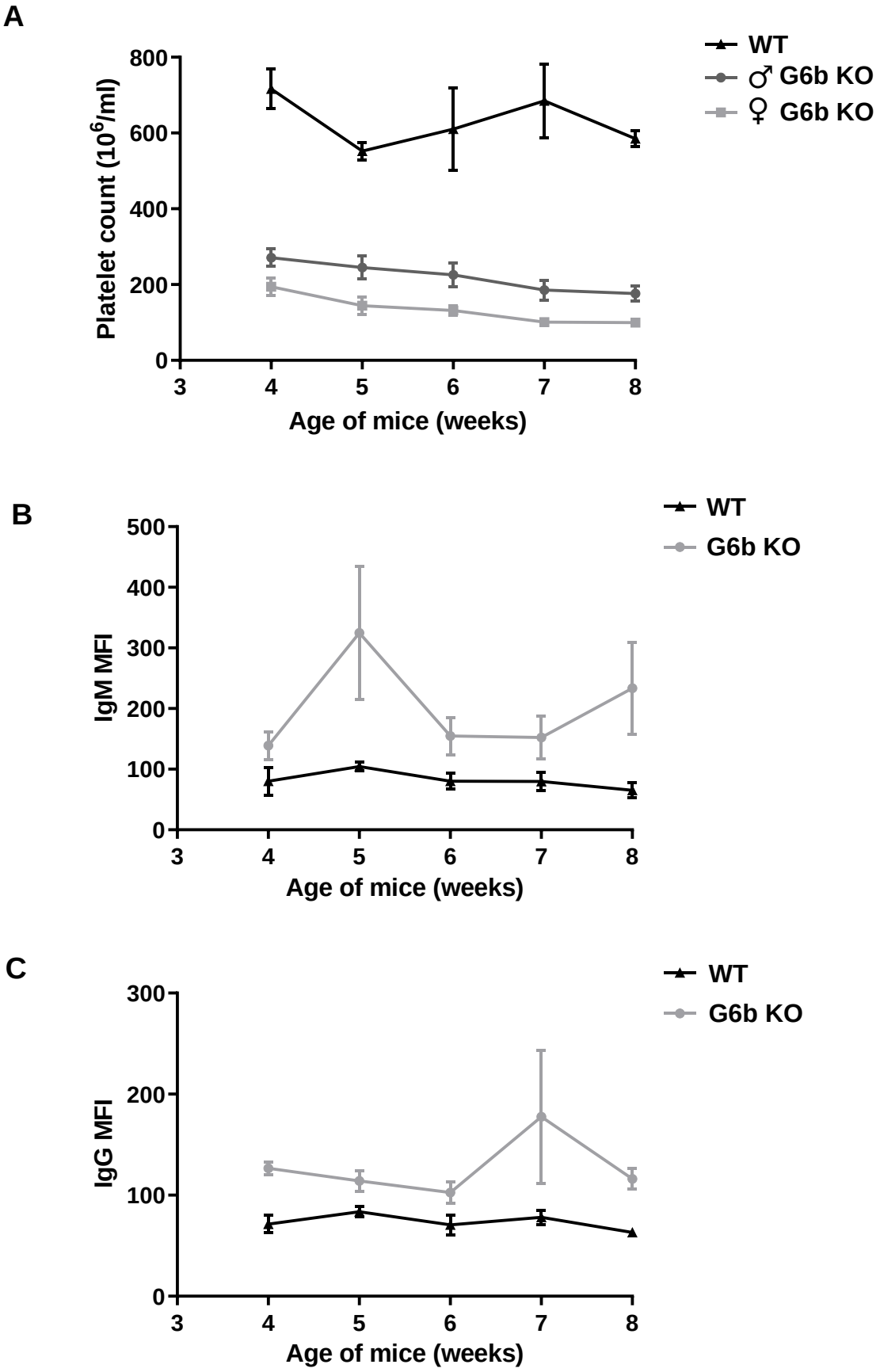


Figure 2

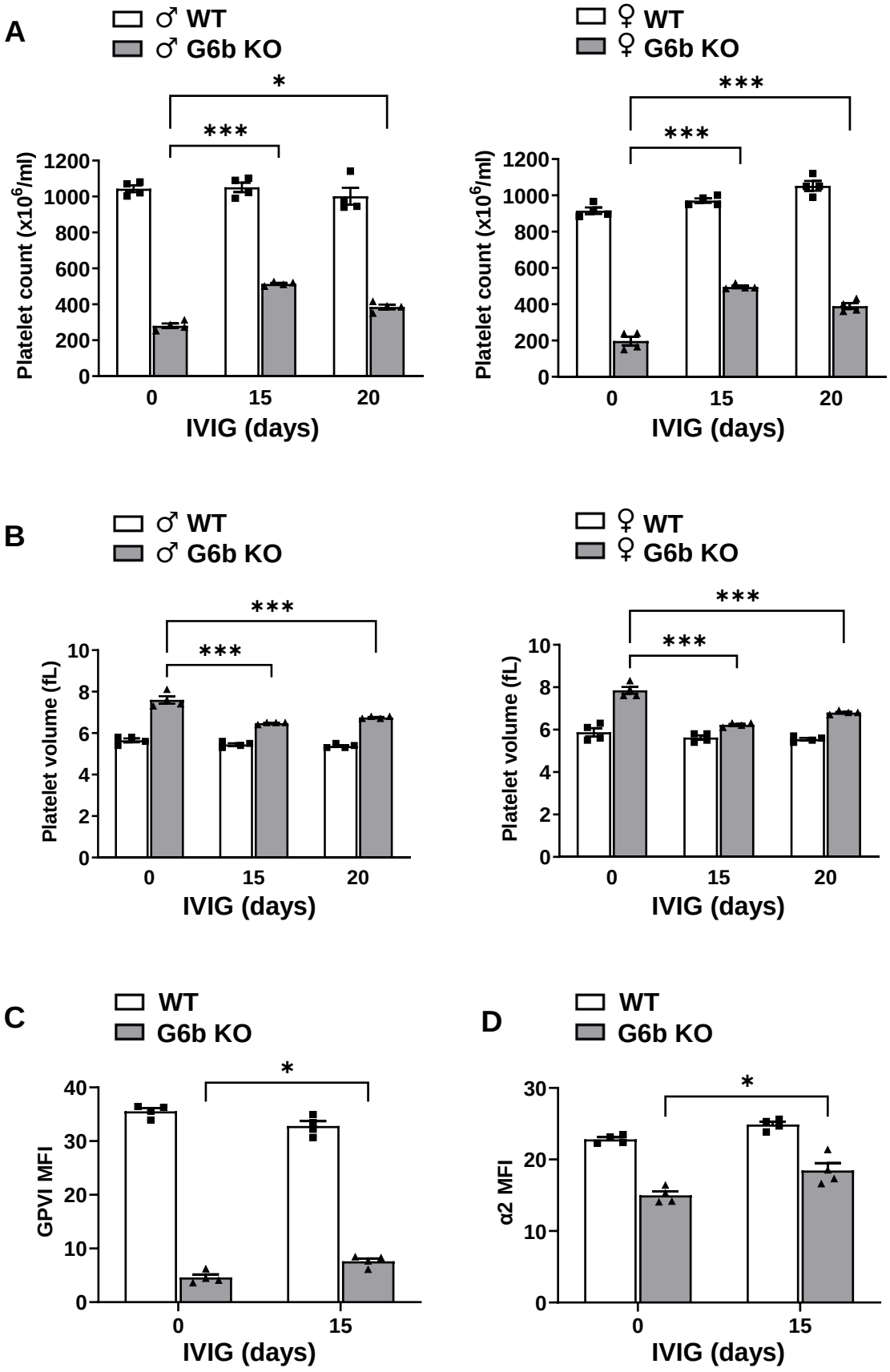


Figure 3

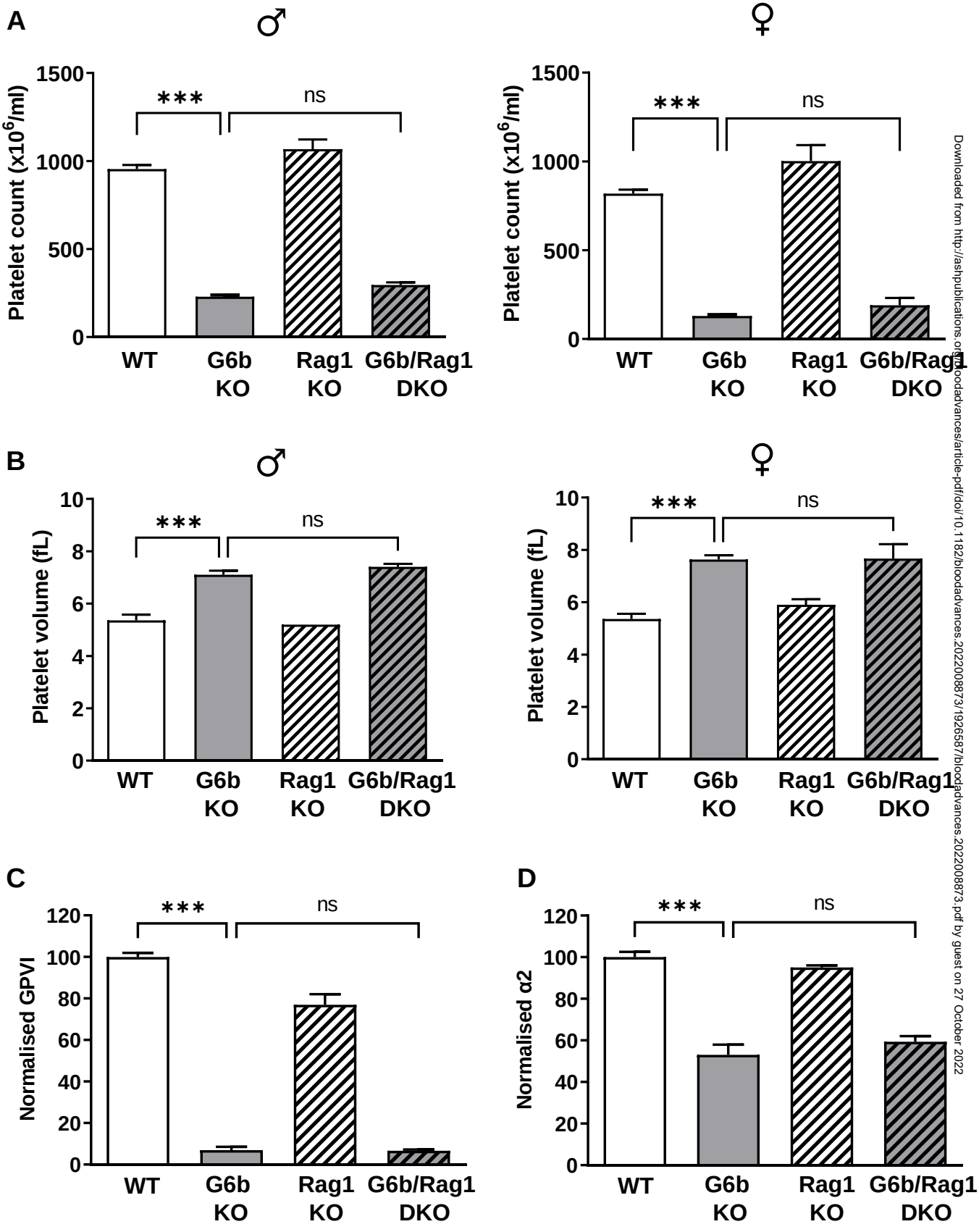
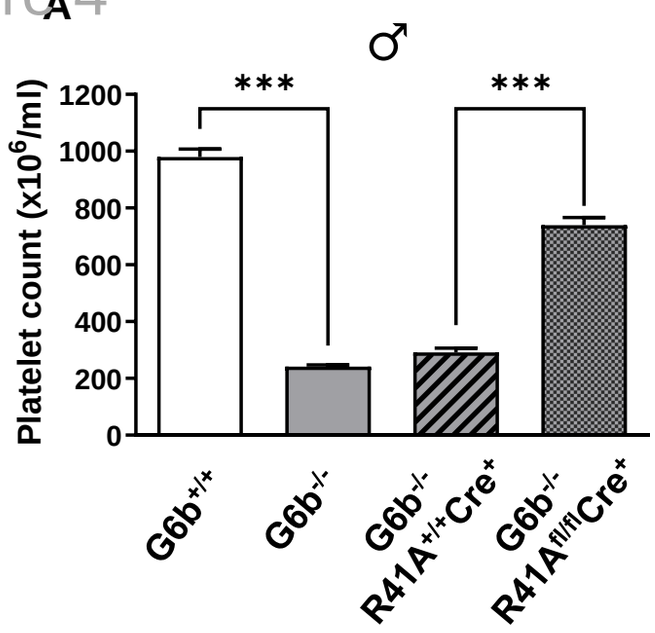
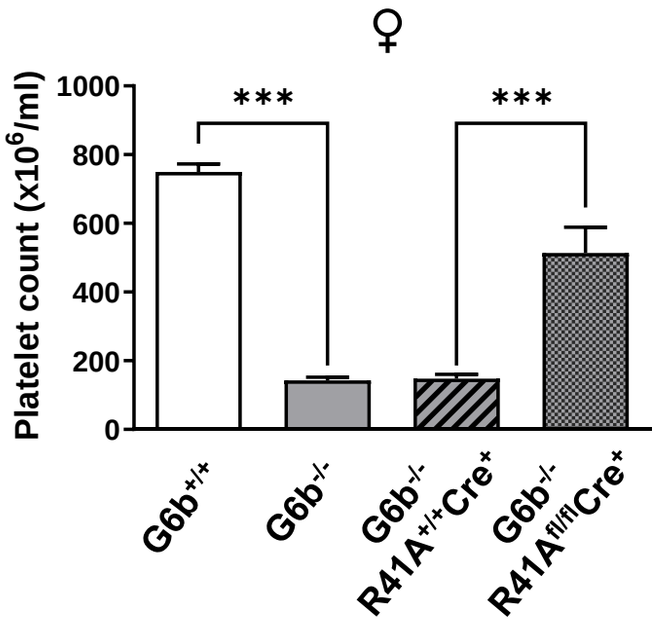
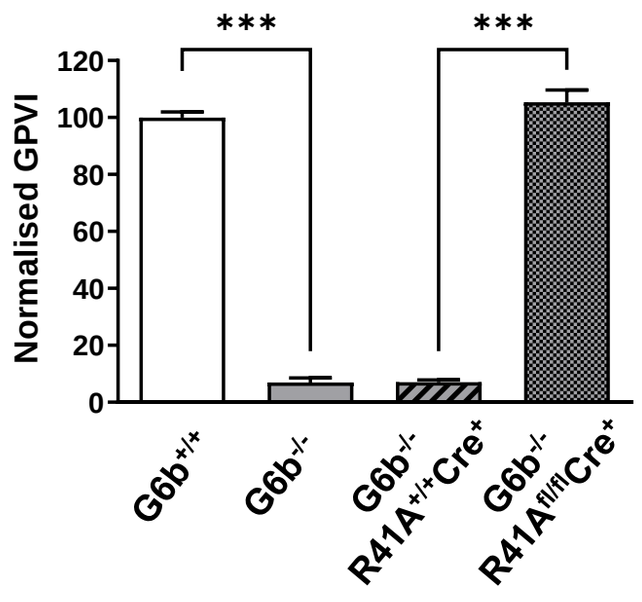
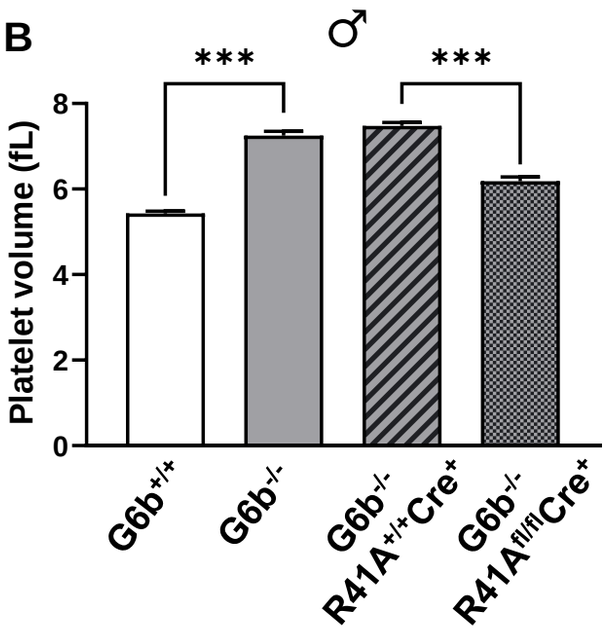


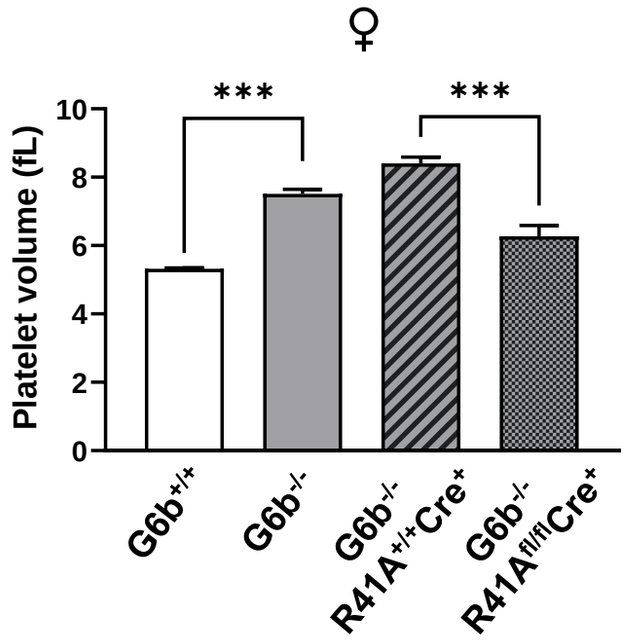
Figure 4

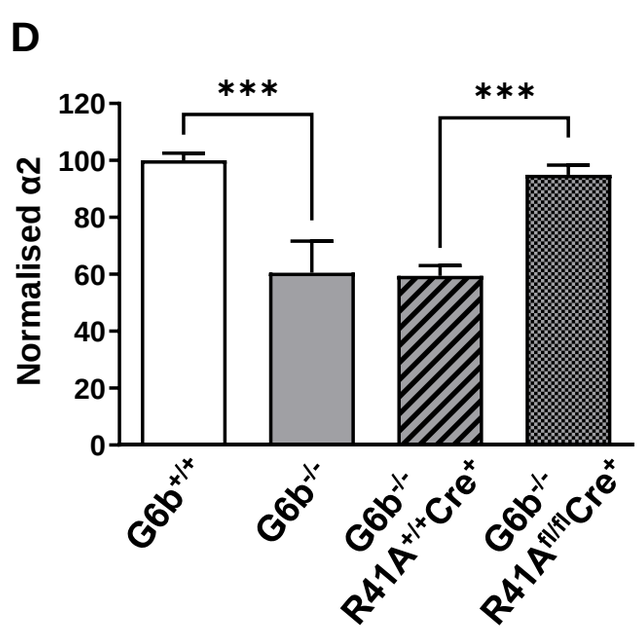


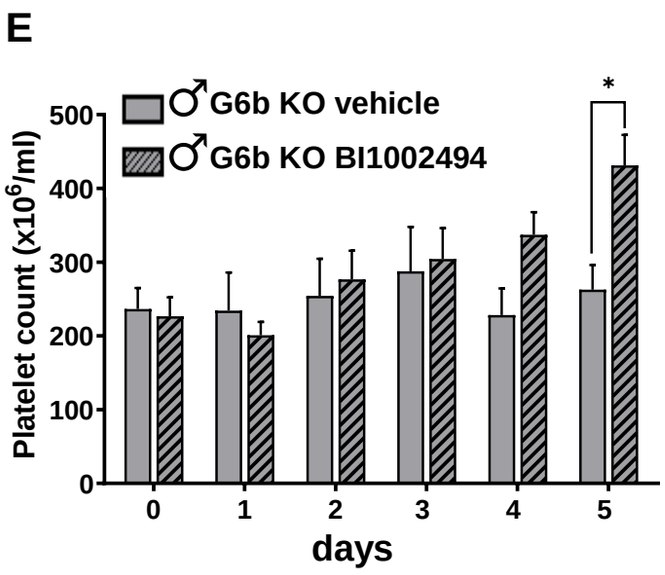


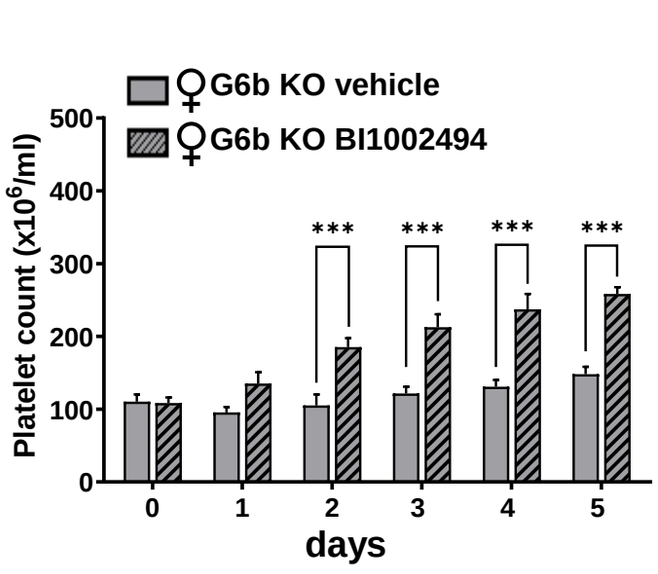


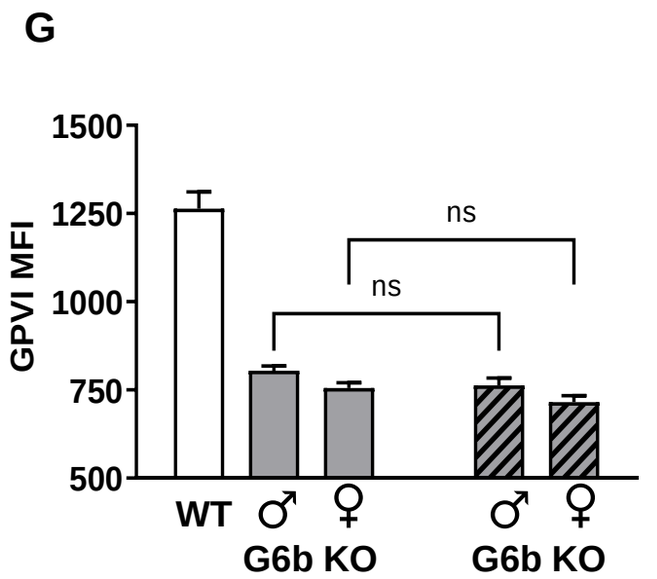


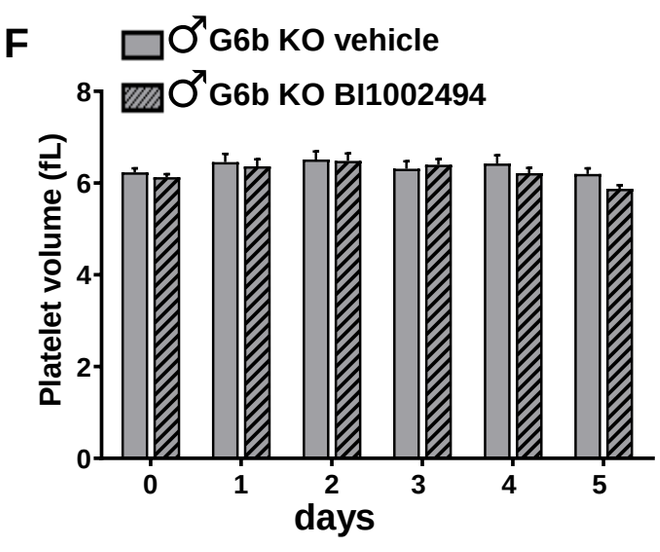


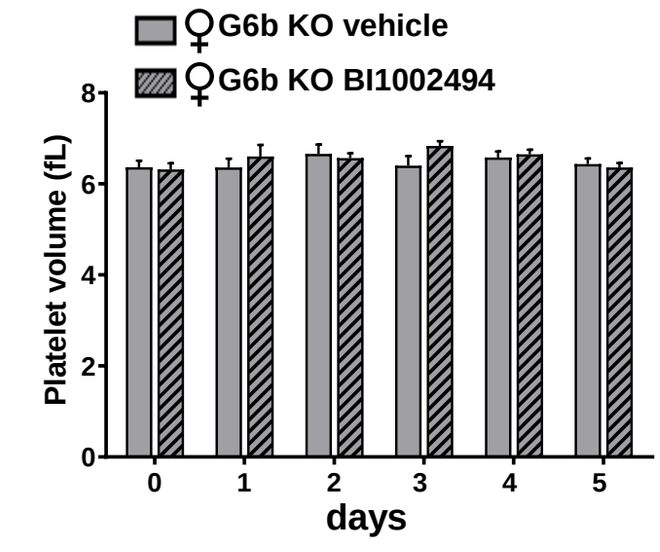


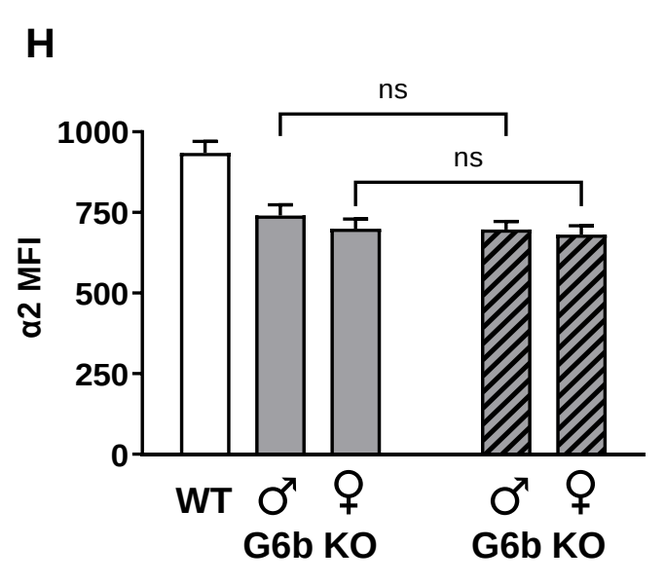


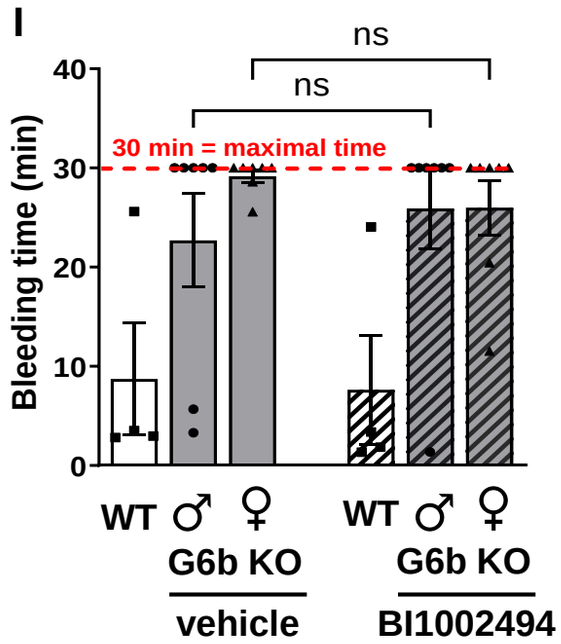












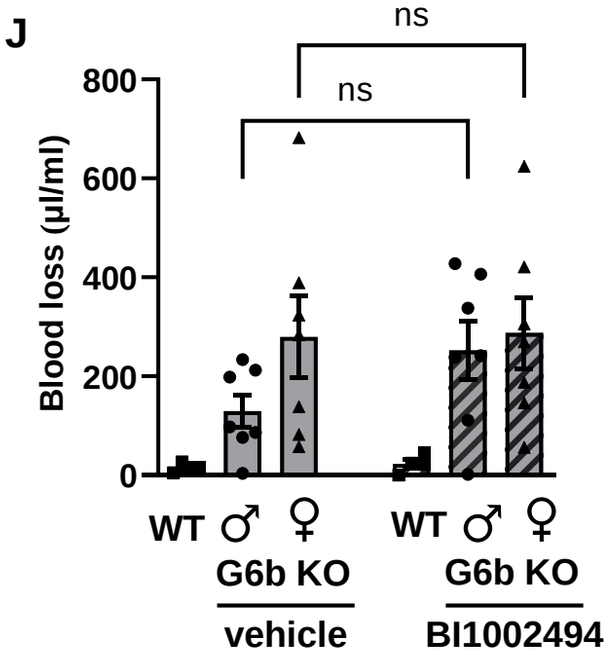


Figure 5

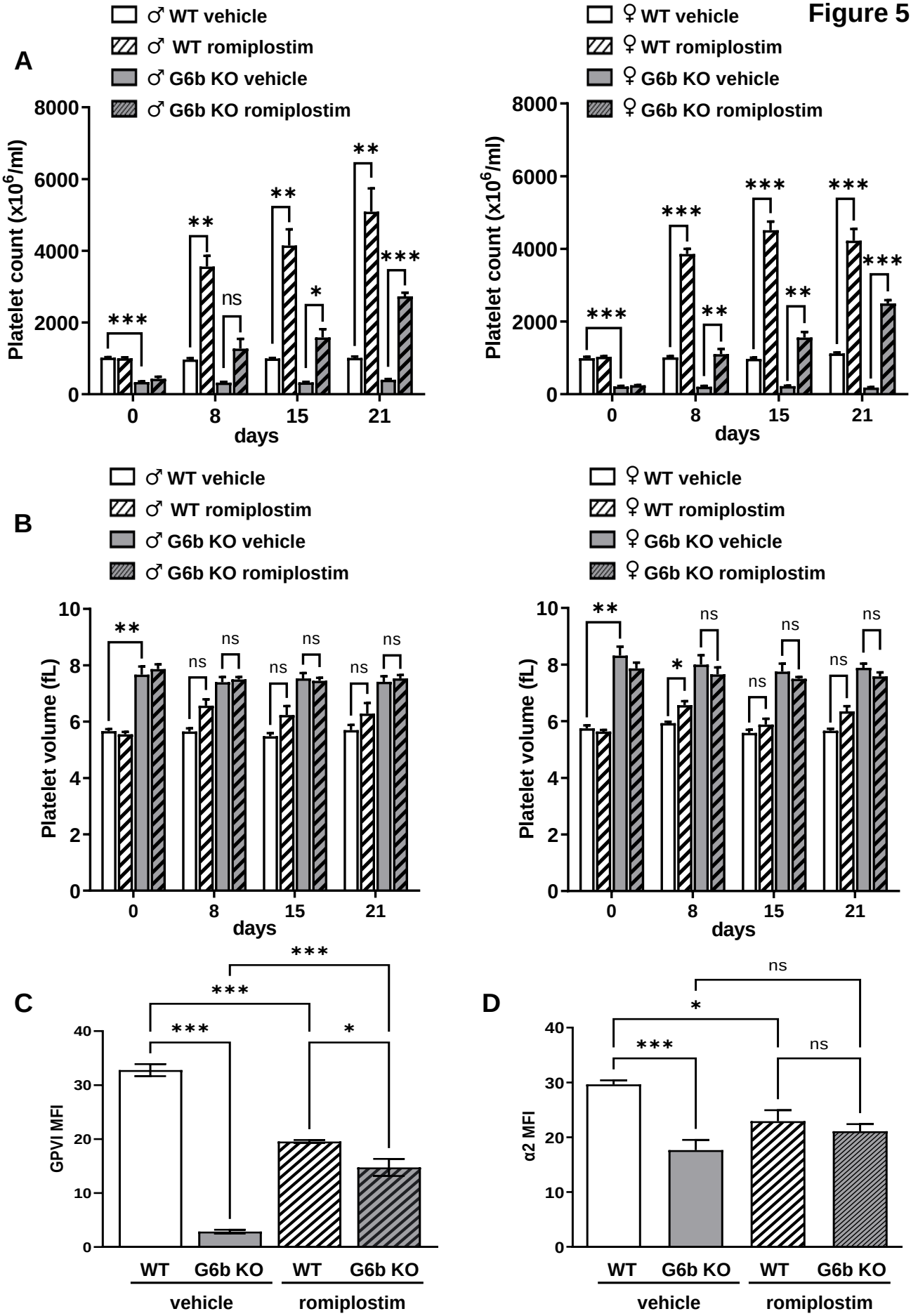


Figure 6

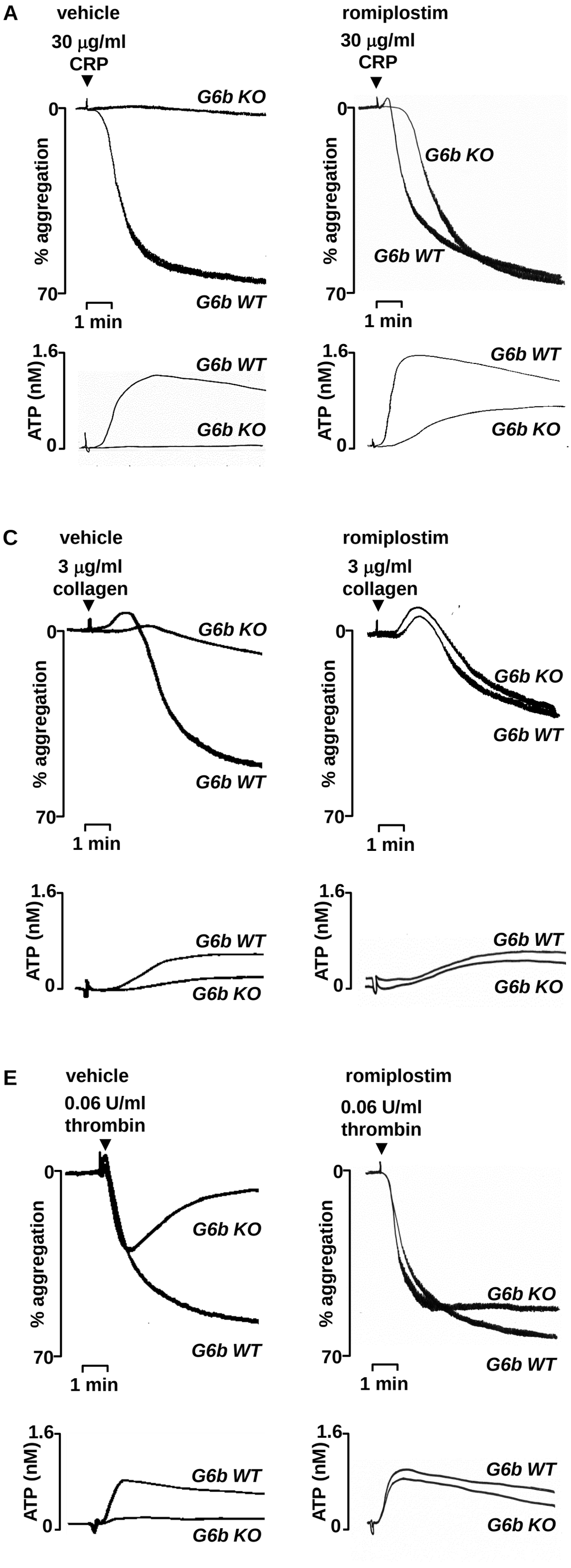


Figure 7