The impact of aberrant von Willebrand factor-GPIbα interaction on megakaryopoiesis and platelets in humanized type 2B von Willebrand disease model mouse

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Abstract

Type 2B von Willebrand disease (VWD) is caused by gain-of-function mutations in von Willebrand factor (VWF). Increased VWF affinity for GPIbα results in loss of high molecular weight multimers and enhanced platelet clearance, both contributing to the bleeding phenotype. Severity of the symptoms vary among type 2B VWD patients, with some developing thrombocytopenia only under stress conditions. Efforts have been made to study underlying pathophysiology for platelet abnormalities, but animal studies have been limited because of species specificity in the VWF-GPIbα interaction. Here, we generated a severe form of type 2B VWD (p.V1316M) knockin mice in the context of human *VWF* exon 28 (encoding A1 and A2 domains) and crossed them with human GPIbα transgenic strain. Heterozygous mutant mice recapitulated the phenotype of type 2B VWD in autosomal dominant manner and presented severe macrothrombocytopenia. Of note, platelets remaining in the circulation had extracytoplasmic GPIbα shed-off from the cell surface. Reciprocal bone marrow transplantation determined mutant VWF produced from endothelial cells as the major cause of the platelet phenotype in type 2B VWD mice. Moreover, altered megakaryocyte maturation in the bone marrow and enhanced extramedullary megakaryopoiesis in the spleen were observed. Interestingly, injection of anti-VWF A1 blocking antibody (NMC-4) not only ameliorated platelet count and GPIbα expression, but also reversed MK ploidy shift. In conclusion, we present a type 2B VWD mouse model with humanized VWF-GPIbα interaction which demonstrated direct influence of aberrant VWF-GPIbα binding on megakaryocytes.

Introduction

Von Willebrand disease (VWD) is a common inherited bleeding disorder caused by defects in the function or synthesis of von Willebrand factor (VWF).[1] VWD is classified into three primary categories: type 1, type 2, and type 3. Among them, type 2B VWD represents variants with increased affinity for platelet GPIbα, which is paradoxically associated with bleeding symptoms, not thrombosis.[2,3] It has been known that enhanced binding of gain-of-function mutant VWF to platelets and accelerated clearance, in combination with higher susceptibility to ADAMTS13, result in loss of the largest VWF multimers and thrombocytopenia.[2-4] Of note, clinical manifestations and results of laboratory tests are heterogeneous among type 2B VWD patients, depending on the mutations involved.[5] In addition, one unsolved issue in the pathophysiology of type 2B VWD is the regulatory mechanism of aberrant VWF A1-GPIbα on megakaryocytes (MK) and thrombopoiesis. In addition to accelerated platelet clearance, impaired megakaryopoiesis have also been implicated as the cause of thrombocytopenia.[6,7]

To date, limited research of VWD using mouse models have been reported due to species-incompatibility of VWF A1-GPIbα interaction. Previous efforts have been made to overcome species-specificity in VWF A1-GPIbα interaction.[8] However, later study found that single amino acid substitutions may not be sufficient to fully convert the species-specific binding property,[9] suggesting that replacement of the entire VWF A1 is desired to mimic human

VWF A1-GPIbα interaction *in vivo*. In addition, mouse VWF A1-GPIbα interaction was found not to be identical to that of human.[10-12] In this study, we report the first mouse model of type 2B VWD having gain-of-function mutation in humanized VWF A1-GPIbα interaction. We have employed the strategy to target *Vwf* exon 28 to humanize VWF A1/A2 domains,[13] and generated type 2B VWD model by mutagenizing the human VWF A1 with the p.V1316M substitution. This strain was bred with humanized GPIbα transgenic strain to humanize VWF A1-GPIbα interaction *in vivo*. As expected, this model closely recapitulated platelet phenotype of human type 2B VWD in autosomal dominant manner and was proven to be a valuable tool to study the biological effect of VWF A1-GPIbα interaction *in vivo*.

Methods

Animal experiments

All animal procedures were performed in accordance with the National Institute of Health Guideline for the Care and Use of Laboratory Animals. All protocols for animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) of The Scripps Research Institute and Medical College of Wisconsin.

Generation of human von Willebrand factor exon 28 knockin mice with p.V1316M mutation and cross-breeding with human GPIbα transgenic mice

A new knockin strain having type 2B VWD mutation was generated at the Transgenic Core Facility, Blood Research Institute/Medical College of Wisconsin. The targeting vector previously used to generate a knockin mouse with *Vwf* exon28 replaced by human homolog (VWF^{hA1}) was used with additional mutagenesis of p.V1316M in the A1 domain.[13] Correctly targeted embryonic stem cells were injected into blastocyst stage embryos to generate chimeric mice. In order to remove the loxP-flanked Neo cassette, these mice were bred with B6.FVB-Tg (EIIa-Cre) C5379Lmgd/J mice (The Jackson Laboratory) and then crossed with the human GPIbα transgenic mouse strain in which platelets express only human GPIbα in the GPIb-IX-V complex. The knockin strain having human *VWF* exon 28 with p.V1316M substitution was bred into homozygous (VWF^{2Bhomo} hGPIbα). VWF^{2Bhomo} hGPIbα strain was bred with previously generated VWF^{hA1} hGPIbα strain to generate VWF^{2Bhet} hGPIbα mice having heterozygous p.V1316M mutation.

Platelet count analysis

For blood cell counting, blood obtained by retro-orbital bleeding were analyzed using the IDEXX ProCyte™ (IDEXX Laboratories Inc., Westbrook, ME).

NMC-4 injection study

Monovalent Fab fragment[14] expressed in *D. melanogaster* S2 cells was purified from culture supernatant and administered (2 mg/kg) by retro-orbital vein injection for 4 consecutive days.

Bone marrow hematopoietic stem cell transplantation

Bone marrow (BM) cells were collected from femurs and tibia of donor mice, and BM mononuclear cells (BM MNC) were isolated using ficoll as described previously.[15,16] Six- to 8-week-old recipient mice were conditioned with a lethal dose of 1,100 cGy total body irradiation using a Gammacell 40 Exactor cesium irradiator. Twenty-four hours after irradiation, a dose of 1×10^7 BM MNC was infused by retro-orbital vein injection. Recipients were analyzed beginning at 6 weeks after transplantation.

Plasma von Willebrand factor analysis

VWF antigen (VWF:Ag) was measured by enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-human VWF polyclonal antibody (IgG) cross-reacting with mouse VWF (produced at the Scripps Research Institute).[16] Normal pooled plasma from C57BL/6J wild-type (WT) mice was used as a reference and defined as 1 U/mL. VWF multimers were analyzed by electrophoresis through 1% HGT(P) agarose containing 0.1% sodium dodecyl sulfate followed by western blotting with the same antibodies used for ELISA.[13]

Flow cytometry analysis

Mouse blood samples were collected from the retro-orbital plexus using sodium citrate as anti-coagulant. Blood samples were diluted in phosphate-buffered saline (pH 7.4) containing 2 mM ethylenediaminetetraacetic acid. Samples were stained with anti-CD41 (integrin αIIb) (MWReg30, Biolegend, San Diego, CA), anti-GPIbα (LJ-P3 for hGPIbα, 5A7 for mGPIbα[17], MERU-VasImmune, San Diego, CA), anti-VWF [polyclonal antibodies[13]]). Samples were analyzed using a NovoCyte flow cytometer (ACEA Biosciences). The results were analyzed with FlowJo v.10.7.1.

Results

Generation and characterization of type 2B von Willebrand disease model mice with humanized von Willebrand factor A1-GPIbα interaction

We have previously generated a mouse strain expressing human VWF A1/A2 in the context of mouse VWF and human GPIbα (VWF^{hA1} hGPIbα).[13] In the current study, the same strategy was employed to generate a strain having type 2B VWD mutation (p.V1316M) in human *VWF* exon 28 (encoding A1/A2 domains). The substitution p.V1316M

was chosen as the mutation associated with the most severe bleeding and thrombocytopenia with morphological abnormalities in patients with type 2B VWD.[5] A knockin strain having p.V1316M mutation in human *VWF* exon 28 was cross-bred with transgenic mice expressing human GPIbα on platelets[13,18] The mutant VWF allele (p.V1316M) was maintained either in heterozygous (VWF^{2Bhet} hGPIbα) or homozy-gous (VWF^{2Bhomo} hGPIbα) status, and a list of the mouse strains used in the current study is shown in Figure 1A. One of our interests in generating type 2B VWD model mice was whether they present macrothrombocytopenia in autosomal dominant manner as observed with human patients. As expected, platelet counts were significantly decreased in both VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice compared to VWF^{hA1} hGPIbα mice (Figure 1B). Mice having homozygous p.V1316M mutation with mouse GPIbα (VWF^{2Bhomo} mGPIbα) showed normal platelet count, confirming that

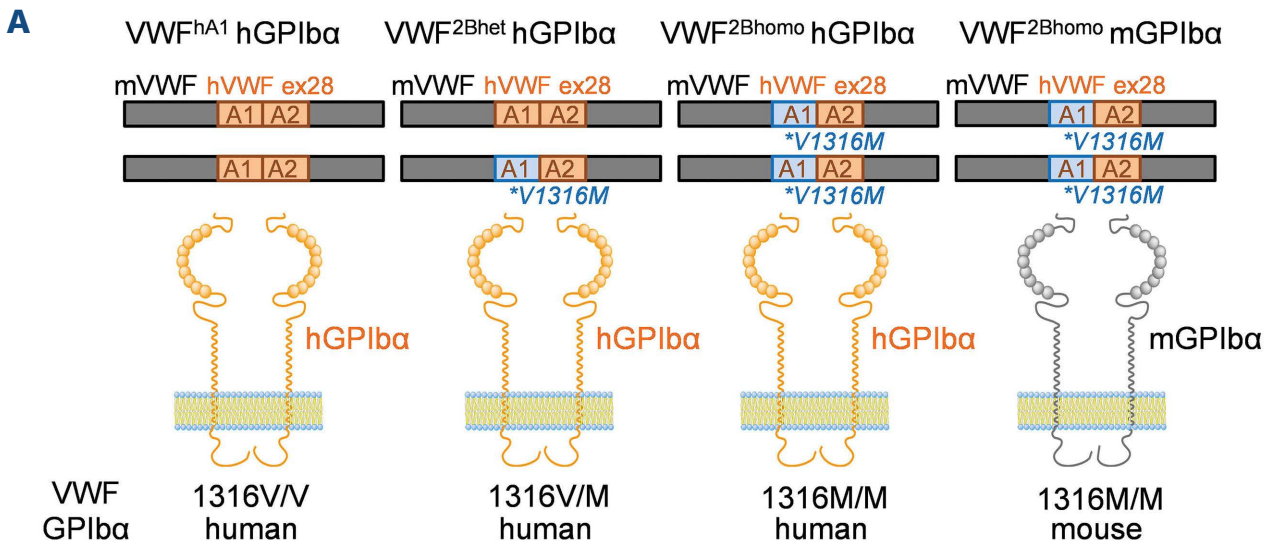

Figure 1. Generation of a mouse model of type 2B von Willebrand disease with humanized von Willebrand factor A1-GPIbα interaction. (A) A knockin strain having human von Willebrand factor (*VWF*) exon 28 (encoding A1 and A2) bred with human GPIbα transgenic strain (VWF^{hA1} hGPIbα) has been previously described.[13] A new knockin strain of human *VWF* exon28 with p.V1316M mutation has been generated in the background of mouse GPIbα (VWF^{2Bhomo} mGPIbα) and crossed with human GPIbα transgenic strain, and maintained either heterozygote or homozygote (VWF^{2Bhet} hGPIbα or VWF^{2Bhomo} hGPIbα, respectively). These 4 mouse strains were analyzed for platelet count (B), plasma VWF (C), and VWF multimer distribution (D). In (C), males and females are depicted as black and red dots, respectively. (E) VWF^{hA1} hGPIbα, VWF^{2Bhet} hGPIbα, and VWF^{2Bhomo} hGPIbα mice (n=5 in each strain) were analyzed by tail bleeding time assay. VWF^{2Bhet} hGPIbα mice treated with intravenous NMC-4 administration (2 mg/kg daily for 4 consecutive days, n=6) were also tested by tail bleeding time assay on the fourth day. Statistical analysis was performed by Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. ***P*<0.01, ****P*<0.001.

thrombocytopenia is caused by gain-of-function VWF mutation only when species compatible GPIbα is expressed. Platelet size was increased in both VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice (*Online Supplementary Figure S1A*). Decrease in the levels of VWF in plasma might be attributed to spontaneous binding to platelet GPIbα and enhanced clearance of high molecular weight (HMW) VWF multimers. In support of this idea, VWF multimer analysis showed loss of HMW VWF multimers in the plasma of VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice, the effect was more profound in the latter (Figure 1D). In addition, increased susceptibility of type 2B mutant VWF to ADAMTS13 has been previously reported[4,10] and this mechanism may also contribute to loss of HMW VWF multimers in our mice. Interestingly, plasma VWF level was also decreased in VWF^{2Bhomo} mGPIbα mice. In this strain, gain-of-function VWF has minimum spontaneous binding to mGPIbα expressed on platelet surface, and therefore, loss of HMW VWF multimer was less obvious compared to the strains with hGPIbα. Decreased plasma VWF in this strain may be, at leaset partly, caused by impaired VWF synthesis, secretion or accelerated degradation. Consistently, plasma VWF level of VWF^{2Bhomo} hGPIbα mice was lower than that of VWF^{2Bhet} hGPIbα mice despite similar severity of thrombocytopenia, indicating the presence of additional mechanism for reduced VWF beyond spontaneous binding and clearance. As expected, both VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice showed prolonged tail bleeding time which did not stop in 600 seconds (n=5 in each strain, Figure 1E).

Activation of circulating platelets in VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice

In order to characterize circulating platelets of VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice, blood samples were analyzed by flow cytometry. In both strains, the expression of hGPIbα on platelet surface was markedly reduced, and VWF^{2Bhomo} hGPIbα mouse was more severely affected than VWF^{2Bhet} hGPIbα mice (Figure 2A). A small proportion of platelets with preserved hGPIbα expression were found in circulation of VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice with bound VWF on their surface (Figure 2A, upper right quadrant). There was not obvious GPIbα cleavage or VWF binding observed with platelets from mice having homozygous p.V1316M mutation with mouse GPIbα (VWF^{2Bhomo} mGPIbα), confirming that GPIbα cleavage is triggered by spontaneous binding of gain-of-function mutant VWF (Figure 2B). Western blotting of plasma samples confirmed cleavage of hGPIbα, glycocalicin, was abundantly present in VWF^{2Bhomo} hGPIbα mice and VWF^{2Bhet} hGPIbα mice (Figure 2C). GPVI is a collagen receptor expressed on platelets and is also known to be proteolytically cleaved in response to activation stimuli such as

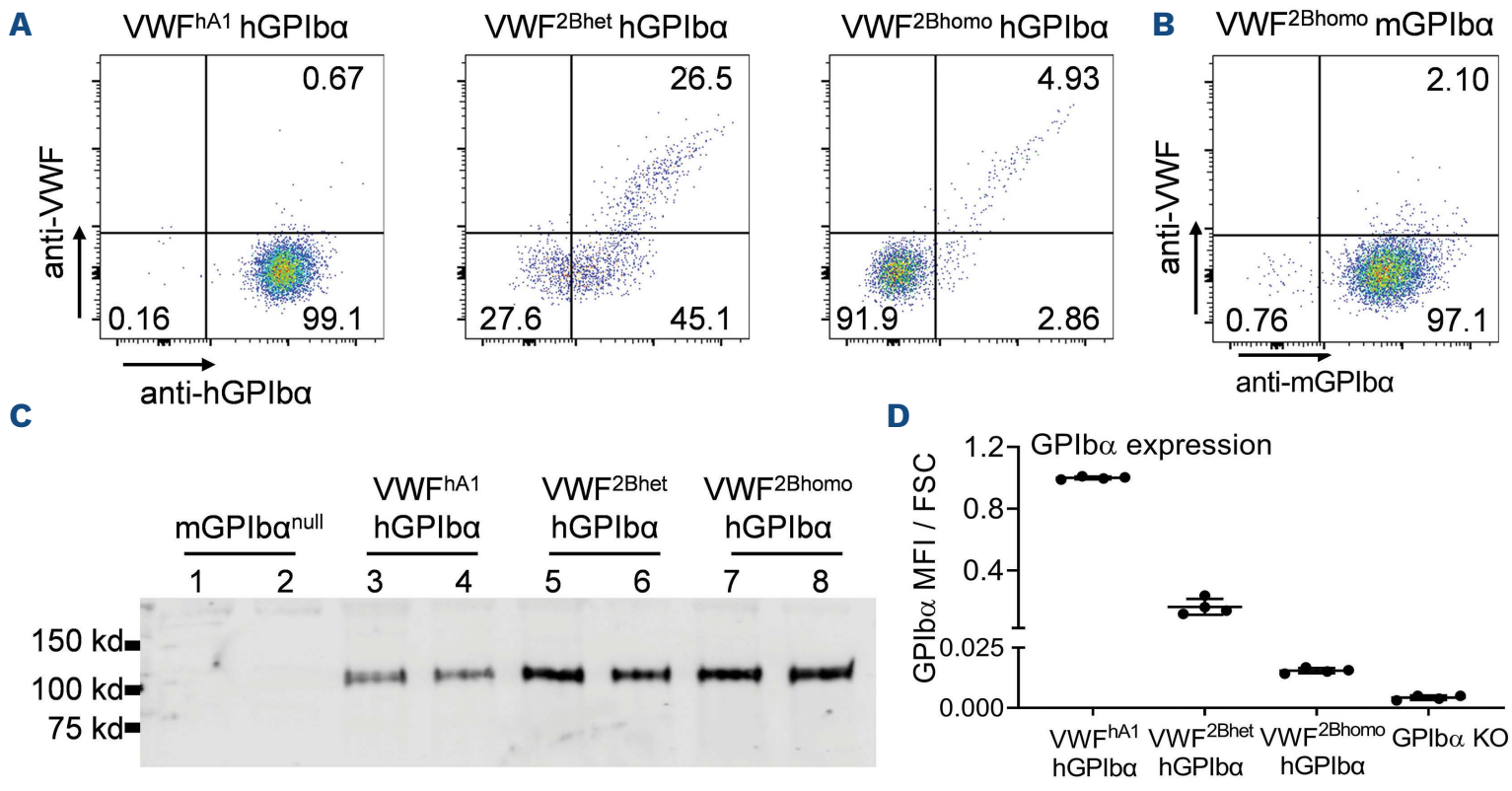

Figure 2. Platelet hGPIbα is cleaved off from platelets in type 2B von Willebrand disease model mice. (A and B) Whole blood samples collected from each strain were stained with AlexaFluor 488 labeled anti-hGPIbα antibody (LJ-P3) or anti-mGPIbα antibody (5A7) and AlexaFluor 647 labeled von Willebrand factor polyclonal antibody (anti-VWF). After staining, blood cells were analyzed by flow cytometry. (C) Plasma samples collected from 2 mice of each strain were analyzed by sodium dodecyl sulfate gel electrophoresis followed by western blotting. PVDF membrane was probed with anti-glycocalicin polyclonal antibody, and the signal was detected with IRDye 800 goat anti-rabbit IgG. The image was obtained using LI-COR Odyssey imaging system. (D) Mean fluorescence intensity (MFI) of hGPIbα signal analyzed by flow cytometry (n=4 in each strain) was normalized by forward side scatter (FSC) MFI to cor-rect platelet size varied among strains.

ligand engagement, elevated shear, and coagulation.[19] The expression of GPVI on platelet surface was also decreased in VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice compared to VWF^{hA1} hGPIbα mice (*Online Supplementary Figure S1B*). Western blotting of VWF^{2Bhomo} hGPIbα mouse platelet lysate confirmed loss of full-length hGPIbα, leaving C-terminal fragment after shedding *(Online Supplementary Figure S1C*). Interestingly, the amount of intracellular filamin A (FlnA) was decreased in VWF^{2Bhomo} hGPIbα mouse platelets compared to VWF^{hA1} hGPIbα mouse platelets. The level of FlnA was also quantified by staining platelets intracellularly after fixation and permeabilization, followed by platelet size correction (*Online Supplementary Figure S1D*). Reduced FlnA content in VWF^{2Bhomo} hGPIbα mouse platelets was confirmed by flow cytometry, indicating activation of μ-calpain and enhanced FlnA degradation in VWF^{2Bhomo} hGPIbα mouse platelets.[20,21] Functional defect of platelets in response to agonist stimulation has been reported in patients and mouse model of type 2B VWD.[22-24] Thus, mouse platelets have been stimulated with PAR4 activating peptide (PAR4-AP) and fibrinogen binding was evaluated by flow cytometry. As expected, fibrinogen binding was impaired in VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mouse platelets compared to VWF^{hA1} hGPIbα mouse platelets (*Online Supplementary Figure S2*). Defect in fibrinogen binding following agonist stimulation has also been previously reported in a mouse model of platelet-type VWD.[25]

Contribution of megakaryocyte/platelet versus endothelial cells to the pathophysiology of type 2B von Willebrand disease

Plasma VWF is primarily derived from endothelial cells (EC), and platelets contain 10-15% of total blood VWF.[1] Thus, one could postulate that the pathology of type 2B VWD is caused by endothelial synthesis of mutant VWF. Previous study showed that VWF spontaneously bound to the surface and intracellular canals of cultured MK derived from patients with type 2B VWD.[6] This result suggests a possibility that mutant VWF binds to GPIbα during synthesis in MK, leading to impaired platelet generation. Thus, reciprocal BM transplantation between VWF^{hA1} hGPIbα and VWF^{2Bhomo} hGPIbα mice was performed. VWF^{hA1} hGPIbα mice lethally irradiated and transplated with VWF^{2Bhomo} hGPIbα BM hematopoietic stem cells (HSC) expressed VWF with normal human A1 sequence in EC and gain-of-function mutant VWF (p.V1316M) in MK/platelets (2B^{MK}, Figure 3A). Conversely, VWF^{2Bhomo} hGPIbα mice irradiated and transplanted with VWF^{hA1} hGPIbα BM HSC expressed mutant VWF in EC and normal VWF in MK/platelets (2B^{EC}). VWF^{hA1} hGPIbα mice irradiated and transplanted with allogenic VWF^{hA1} hGPIbα mouse BM HSC were prepared as controls. Platelet count analyzed after BM reconstitution showed marked thrombocytopenia in 2B^{EC} (Figure 3B). Platelet count in 2B^{MK} mice was lower than control VWF^{hA1} hGPIbα mice but the difference did not reach statistical significance. Flow cytometry

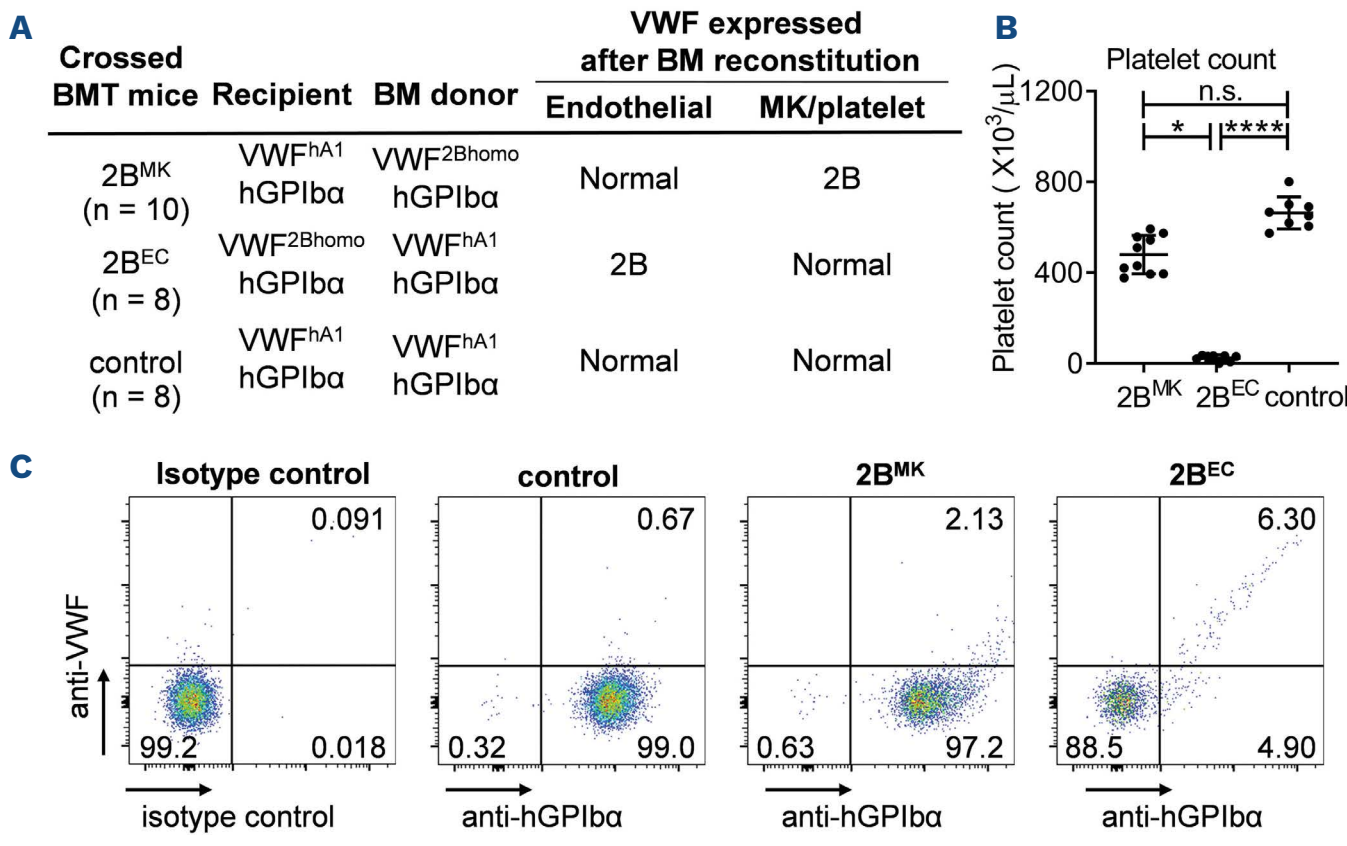

Figure 3. Crossed bone marrow transplantation between VWF^{hA1} hGPIbα and VWF^{2Bhomo} hGPIbα mice. (A) In order to express type 2B von Willebarnd factor (VWF) selectively in endothelial cells (EC) or megakaryocytes[MK]/platelets, VWF^{hA1} hGPIbα and VWF^{2Bhomo} hGPIbα mice were lethally irradiated (1,100 cGy) and transplanted with hematopoietic stem cells (HSC) derived from the other strain. (B and C) Platelet count and platelet flow cytometry analyses. Surface expression of GPIbα and VWF binding were analyzed using the method described in Figure 2A after bone marrow (BM) reconstitution. Data are shown as mean ± standard deviation. Statistical analysis performed with the Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. *P<0.05, ****P<0.0001.

analysis showed that hGPIbα was shed off from the surface of the $2B^{EC}$ mouse platelets (Figure 3C). The expression of hGPIbα was preserved and platelets with spontaneously bound VWF was not abundantly present in $2B^{MK}$ mice (Figure 3C, upper right quadrant). These results demonstrate that severe thrombocytopenia and hGPIbα cleavage observed with type 2B VWD model mice are primarily caused by mutant VWF synthesized in EC.

Enhanced megakaryocyte maturation in the bone marrow and extramedullary megakaryopoiesis in spleen

Previous studies have indicated abnormal megakaryopoiesis and thrombopoiesis in type 2B VWD. The challenging aspect of MK study using patients' samples is the difficulty in obtaining BM cells for analysis because BM aspirate or biopsy is an invasive procedure especially for patients with a bleeding tendency. Therefore, earlier studies have been performed with MK differentiated *in vitro* from $CD34^+$ cells isolated from patients' peripheral blood.[6,7] In the current study, we have studied newly generated type 2B VWD mouse model to evaluate MK in the BM and extramedullary megakaryopoiesis in other organs. Interestingly, total number of MK in the BM was significantly higher in VWF^{2Bhet} hGPIbα mice compared to VWF^{hA1} hGPIbα mice (Figure 4A). MK ploidy analysis showed increased percentage of high ploidy MK (32N) in VWF^{2Bhet} hGPIbα mice compared to VWF^{hA1} hGPIbα mice (Figure 4B). In order to examine whether increased ploidy observed with VWF^{2Bhet} hGPIbα mouse BM MK is caused by increased thrombopoietin (TPO) level, plasma TPO was measured by ELISA. Plasma TPO levels in VWF^{2Bhet} hGPIbα mice were slightly higher than VWF^{hA1} hGPIbα mice but the difference was not significant (Figure 4C). Dissection of VWF^{2Bhet} hGPIbα mice showed splemomegaly, indicating the presence of extramedullary hematopoiesis (Figure 4D). As expected, immunofluorescence microscopy showed increased number of MK present in the spleen of VWF^{2Bhet} hGPIbα mice (Figure 4E and F).

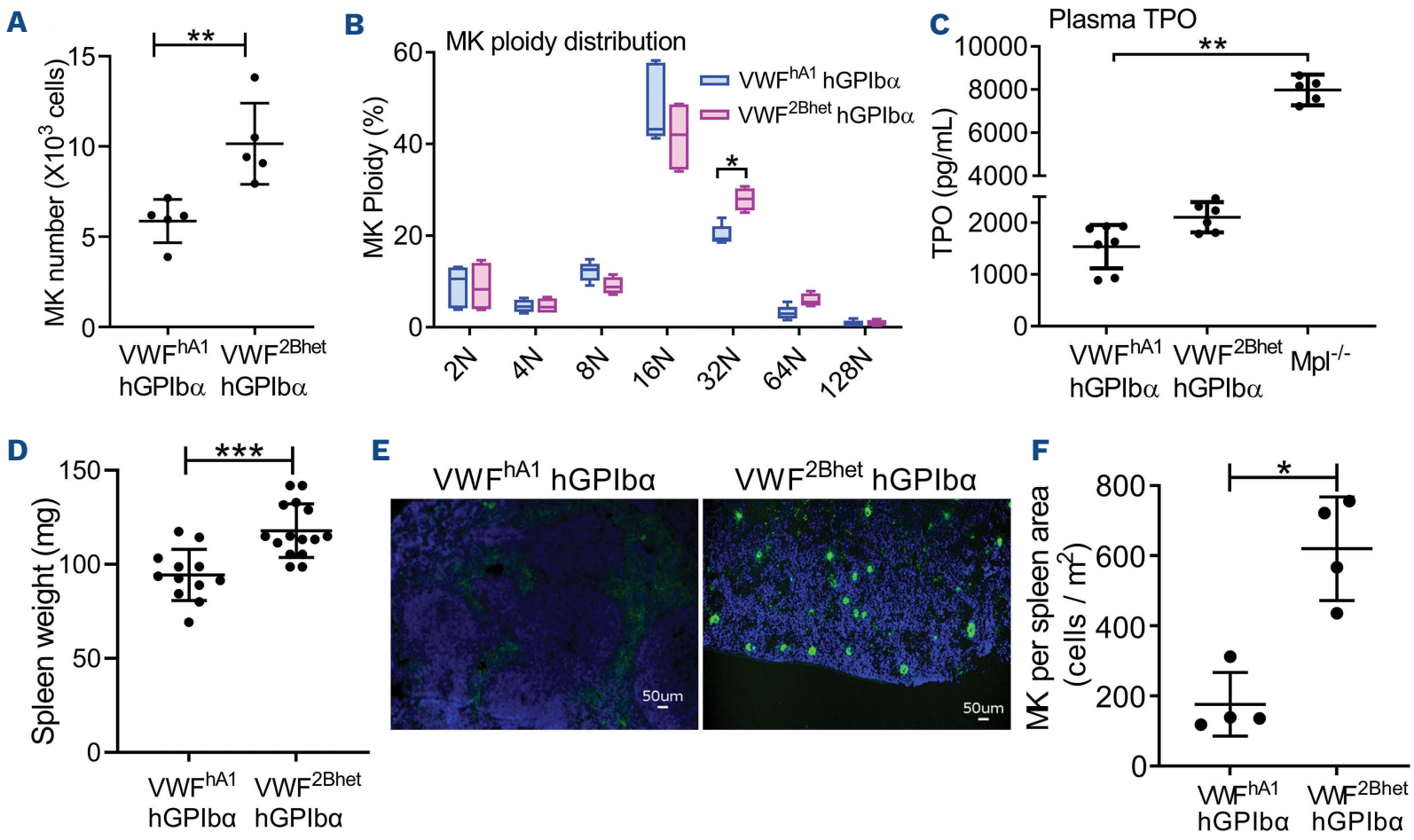

Figure 4. Analysis of megakaryocytes in bone marrow and spleen. (A and B) Bone marrow (BM) cells of VWF^{hA1} hGPIbα and VWF^{2Bhet} hGPIbα mice (n=5 in each group) were flushed from right femurs, stained with Brilliant Violet 421 labeled anti-mouse CD41 antibody and propidium iodide in the presence of RNase A, and analyzed by flow cytometry. Total megakaryocyte (MK) number per femur and MK ploidy distribution are shown. (C) Thyroid peroxidase antibody (TPO) levels in the plasma of VWF^{hA1} hGPIbα (n=7), VWF^{2Bhet} hGPIbα (n=6), and c-$Mpl^{-/-}$ (as controls, n =5) were measured by enzyme-linked immunosorbant assay. (D) Weight of the spleens dissected from VWF^{hA1} hGPIbα (n=12) and VWF^{2Bhet} hGPIbα (n=15) are shown. Females of 9-11 weeks old mice were used to minimize the effect of body size difference. (E and F) Spleens isolated from VWF^{hA1} hGPIbα and VWF^{2Bhet} hGPIbα mice were cryosectioned and immunostained for MK/platelets using rat anti-mouse CD41 antibody and AlexaFluor488-labeled goat anti-rat IgG. Nuclei were counterstained with DAPI. Scale bars =50 μm. The number of MK per spleen area were counted using BZ-X700 Fluorescence microscope (Keyence). Data were shown as scatter plots with mean ± standard deviation or 25^{th}-75^{th} percentile boxes with min-to-max-whiskers. Data were analyzed by Mann-Whitney non-parametric test in (A) and (F), two-way ANOVA with Šidák's multiple comparisons test in (B), Kruskal-Wallis test with Dunn's multiple comparisons test in (C), and unpaired *t*-test in (D). *P<0.05, **P<0.01, ***P<0.001; only significant differences are shown.

Megakaryocytes bind to and internalize gain-of-function mutant von Willebrand factor of endothelial synthesis

One unsolved question was whether MK have a chance to encounter plasma VWF and are influenced by gain-of-function mutant VWF during thrombopoiesis. In order to distinguish VWF of endothelial and MK origin, BM HSC prepared from VWF$^{-/-}$ hGPIbα mice were transplanted into VWF^{hA1} hGPIbα (hA1 MK^{null}) and VWF^{2Bhomo} hGPIbα (2B MK^{null}) to eliminate VWF synthesis in MK (Figure 5A). hA1 MK^{null} and 2B MK^{null} mice express VWF with normal human A1 or p.V1316M mutant A1 sequence only in EC. As expected, 2B MK^{null} presented marked thrombocytopenia and circulating platelets had hGPIbα shed-off from surface (Figure 5B). Immunofluorescent analyses confirmed absence of VWF in the MK of BM and spleen in the hA1 MK^{null} mice (Figure 5C). Interestingly, 2B MK^{null} mice showed weak staining of VWF in the BM MK and strong signal in the splenic MK (Figure 5D). Splenic red pulp harbors an open circulatory system where MK may potentially bind to plasma VWF.[26] Thus, higher VWF staining observed with splenic MK is in agreement with exposure to an open circulatory system. These results show that in the absence of VWF synthesis in MK, MK of 2B MK^{null} mice contain substantial amount of VWF derived from EC synthesis. In order to further characterize platelets generated from these MK, blood samples were fixed, permeabilized, stained with anti-VWF antibody and analyzed by flow cytometry (*Online Supplementary Figure S3*). hA1 MK^{null} platelets had no VWF signal inside because of defective VWF synthesis in MK, and the result was similar to VWF$^{-/-}$ hGPIbα mouse platelets. On the other hand, about half of platelets derived from 2B MK^{null} mice had VWF inside of cells and partially co-localized with hGPIbα signal. These results confirm that MK of type 2B VWD model mice are exposed to and bind to mutant VWF of EC origin. MK with internalized mutant VWF have potential to produce platelets with intracellular VWF and reduced surface GPIbα expression into circulation.

Blocking von Willebrand factor A1-GPIbα interaction inhibits hGPIbα cleavage and ameliorates thrombocytopenia

In order to test if platelet abnormalities and MK ploidy shift observed with VWF^{2Bhet} hGPIbα mice can be reversed by inhibiting VWF A1-GPIbα interaction, anti-VWF A1 blocking antibody (NMC-4) was injected *in vivo*.[27,28] Daily administration of NMC-4 (2 mg/kg, n=6) led to platelet count increase to the levels comparable to VWF^{hA1} hGPIbα mice (Figure 6A). Blocking VWF A1-GPIbα interaction also ameli-

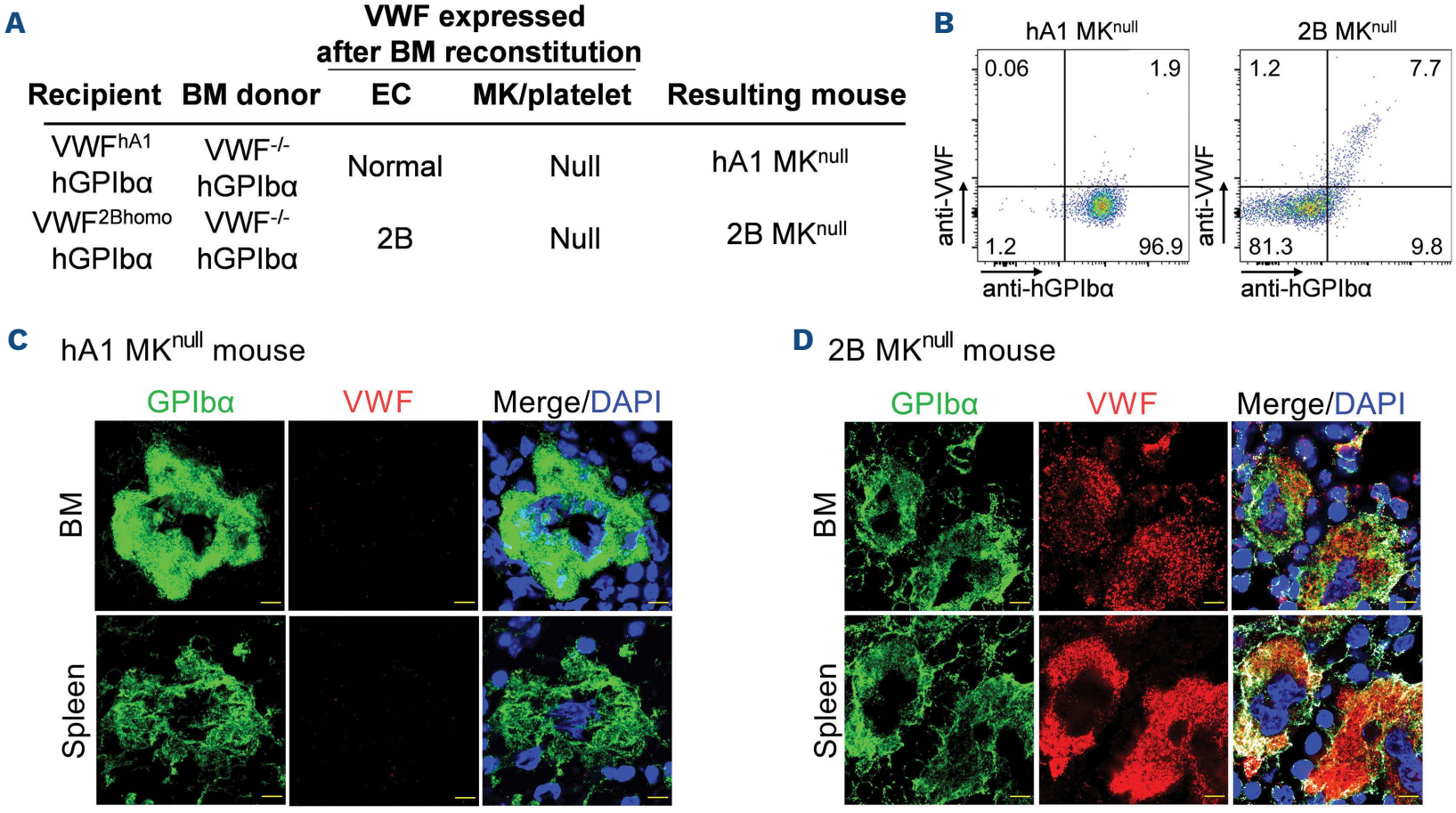

Figure 5. Binding and internalization of gain-of-function mutant von Willebrand factor in megakaryocytes. (A) In order to generate mouse models expressing von Willebrand factor (VWF) only in the endothelial cells (EC), bone marror hematopoietic stem cells (BM HCS) prepared from VWF$^{-/-}$ hGPIbα mice were transplanted into lethally irradiated VWF^{hA1} hGPIbα or VWF^{2Bhomo} hGPIbα mice. Upon BM reconstitution, resulting mice produce normal or gain-of-function mutant VWF only in the EC. (B) Blood samples were collected from hA1 MK^{null} and 2B MK^{null} mice 6 weeks after transplantation. Representative results of platelet flow cytometry analysis are shown. Blood samples were stained with the method described in Figure 2A. (C and D) Femurs and spleen were snap frozen, cryosectioned, and stained for GPIbα (LJ-Ib1, green, MERU-VasImmune), VWF (anti-VWF polyclonal antibody, red), counterstained (DAPI, blue), and analyzed by LSM880 laser scanning confocal microscope (Zeiss). Merged signal of GPIbα (green) and VWF (red) is shown in white. Scale bars =5 μm.

orated hGPIbα shedding and the hGPIbα expression was recovered on VWF^{2Bhet} hGPIbα mouse platelets (Figure 6B). It has been known that NMC-4 blocks VWF binding to GPIbα, and we have previously reported that administration of NMC-4 (0.8 mg/kg) prolongs bleeding time even in mice having normal sequence of humanized VWF exon 28 (VWF^{hA1} hGPIbα).[13] Thus, as predicted, despite correction of thrombocytopenia and hGPIbα expression, tail bleeding time was still prolonged in VWF^{2Bhet} hGPIbα administered with NMC-4 (see Figure 1E). Interestingly, BM MK analysis performed after 4 doses of NMC-4 injection showed that MK ploidy shift observed with VWF^{2Bhet} hGPIbα mice was reversed by anti-VWF A1 blocking antibody (Figure 6C, compare with Figure 4B).

Discussion

Mouse models of type 2B VWD have been described using hydrodynamic injection of mutated mouse VWF expression plasmids into VWF$^{-/-}$ mice.[10,11] In these models, mutations were introduced into mouse VWF cDNA, and expressed ectopically in the liver, not in EC or MK. Subsequently, a knockin mouse harboring a type 2B VWD mutation (p.V1316M) in mouse *Vwf* gene has been described.[12,23] This model enabled physiologic expression in EC and MK and provided valuable information. However, introduction of a single point mutation in the context of mouse *VWF* did not reproduce platelet phenotype in autosomal dominant manner and had to be bred into homozygous, suggesting that mutagenesis in mouse VWF A1 in association with mouse GPIbα may not be identical to that of human. Here, we generated a type 2B VWD model with humanized VWF A1-GPIbα interaction. Using the knockin strategy to replace *Vwf* exon 28 - encoding domains A1 and A2 – with that of the human homologue, we have generated a strain with a mutation (p.V1316M) in the sequence of human VWF A1. Subsequent cross-breeding with hGPIbα transgenic mice successfully established a mouse model of type 2B VWD that present macrothrombocytopenia in autosomal dominant manner. Reciprocal HSC transplantation determined VWF of endothelial synthesis as the cause of platelet abnormalities. An open question is whether ADAMTS13 sensitivity is altered by *Vwf* exon28 replacement encoding A1 and A2 (ADAMTS13 cleavage) domains. Enhanced susceptibility of type 2B VWF by ADAMTS13 has been previously reported and the roles of ADAMTS13-mediated proteolysis in the loss of HMW VWF multimers, regulation of platelet aggregates, and impaired hemostasis have been well established.[4,10] Further study is warranted to explore the alteration of ADAMTS13 sensitivity by humanizing VWF A1/A2 domains in VWF^{hA1} hGPIbα mice and potential contribution to the phenotype of type 2B VWD model mice.

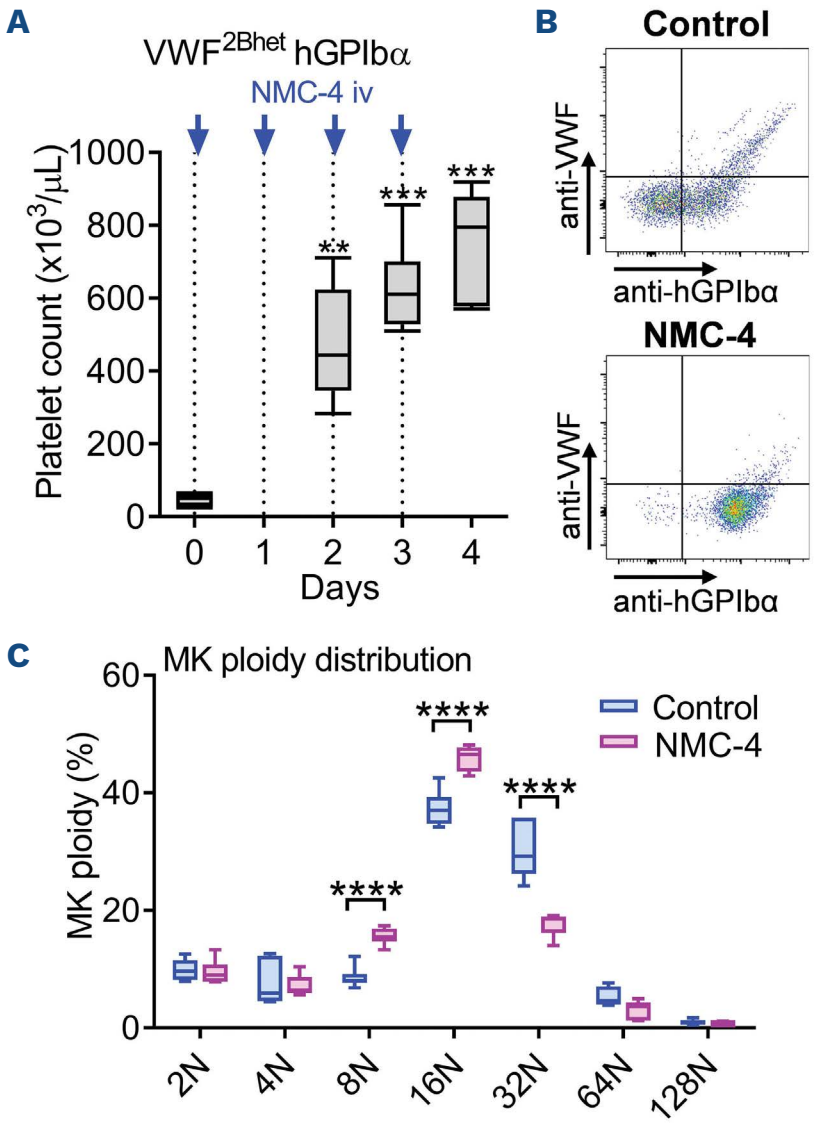

Figure 6. Inhibition of VWF A1-GPIbα reverses the effect of gain-of-function mutant von Willebrand factor on platelets and megakaryocytes. (A) A recombinant monoclonal antibody against von Willebrand factor (VWF) A1 which blocks binding to GPIbα (NMC-4) was intravenously administered into VWF^{2Bhet} hGPIbα mice (2 mg/kg, n=6) daily, and platelet counts were monitored. Data were analyzed by RM one-way ANOVA without assuming sphericity and with Holm-Sidak's post-test for comparing pretreatment values with those at each day post injection. (B) Representative profiles of blood flow cytometry analysis showing recovery of hGPIbα expression on VWF^{2Bhet} hGPIbα mouse platelets after 4 doses of NMC-4 administration. (C) Bone marrow (BM) cells of VWF^{2Bhet} hGPIbα mice treated with daily NMC-4 administration were harvested on day 4 and analyzed by flow cytometry as in the Figure 4B. Data are shown as 25th-75th percentile boxes with min-to-max-whisker and analyzed by two-way ANOVA with Šidák's multiple comparisons test. ***P*<0.01, ****P*<0.001,*****P*<0.0001; only significant differences are shown.

Our type 2B VWD mouse model exhibited exaggerated platelet phenotype compared to human patients. In fact, platelet count in VWF^{2Bhet} hGPIbα mice was about 5% of the control mice with normal VWF A1 sequence (VWF^{hA1} hGPIbα), which was more severe than human patients having the same mutation.[5] In addition, hGPIbα was cleaved from platelet surface, and VWF^{2Bhomo} hGPIbα mice were more severely affected than VWF^{2Bhet} hGPIbα mice. Shedding of mouse GPIbα has also been reported in a study expressing mutant VWF by hydrodynamic gene transfer.[24] In their study, mouse GPIbα cleavage was not reduced when mutant VWF was expressed in mice deficient in ADAM17, indicating ADAM17-independent cleavage.[24] Mechanical cleavage by shear stress caused by engagement of hGPIbα binding to VWF A1 may contribute to the shedding of hGPIbα. Shedding of hGPIbα from the platelet surface has not been previously documented in human type 2B VWD patients.[22] Thus, cleavage of hGPIbα observed in type 2B VWD model might be caused by mouse-specific factors. Of note, both VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice presented marked splenomegaly (see Figure 4). In the developing embryo, hematopoiesis occurs in the liver and spleen. In mouse but not in hu-man, the spleen remains as hematopoietic organ throughout the life.[29] In our study, histological analysis revealed abundant presence of MK in the spleen of VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mice, suggesting extramedullary megakaryopoiesis and potential exposure of MK and platelets to plasma VWF in the open circulatory system of spleen. In support of this idea, transplantation of VWF$^{-/-}$ hGPIbα mouse BM into VWF^{2Bhomo} hGPIbα (2B MK^{null}) showed splenic MK bound to and internalized plasma-derived mutant VWF, and presence of VWF-containing platelets in the circulation of 2B MK^{null} mice. Thus, we speculate that the extramedullary megakaryopoiesis in the spleen contributes to exaggerated platelet phenotype observed with our type 2B VWD model mice.

Another interesting finding is that the expression of gain-of-function mutant VWF protein leads to MK ploidy shift to mature population (see Figure 4). Plasma TPO level was not significantly changed in VWF^{2Bhet} hGPIbα compared to VWF^{hA1} hGPIbα, indicating that the ploidy shift was not caused by elevated TPO levels but by direct influence of aberrant VWF binding to MK. In support of this idea, MK ploidy shift observed in VWF^{2Bhet} hGPIbα mouse was reversed by administration of NMC-4 which blocks VWF A1-GPIbα interaction. Taken together, these results demonstrate that aberrant VWF-hGPIbα occurs not only in platelets but also in MK, the latter leads to altered megakaryopoiesis and possibly platelet generation. We have previously reported signaling effects of GPIbα cytoplasmic tail on MK proliferation and maturation.[30] Crosstalk of GPIbα signaling with TPO-Mpl pathway, mediated by 14-3-3ξ and phosphoinositol-3-kinase/Akt (PI3K/Akt) pathways, might be involved in the effect of gain-of-function mutant VWF on MK proliferation and maturation.[30] Further study is required to elucidate the mechanism of gain-of-function VWF A1 on GPIbα signaling.

As to the functional defect of platelets, impaired activation of the small GTPase Rap1 has been previously reported as a cause of thrombocytopathy and bleeding tendency in type 2B VWD model mice.[22] Subsequent study determined preactivation and exhaustion of the PKC pathway as the cause of impaired Rap1 signaling and thrombopathy in type 2B VWD.[24] In addition, the same group identified the effect of dysregulated RhoA/ROCK/LIMK/cofilin pathway on Mk and macrothrombocytopenia.[23] In the current study, we found enhanced degradation of intracellular FlnA, a major substrate for μ-calpain in VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mouse platelets (*Online Supplementary Figure S1*).[21,31] FlnA is a scaffold protein which links cytoskeletal proteins to GPIb-IX in platelets and plays an important role in the regulation of contractile force and production of normal size platelets.[32,33] We have previously reported in a mouse model of sitosterolemia that activation of μ-calpain and enhanced degradation of FlnA led to cytosolic redistribution of FlnA, thereby contributing to generation of large platelets.[21] In the current study, there was a significant population of VWF^{2Bhet} hGPIbα and VWF^{2Bhomo} hGPIbα mouse platelets refractory to agonist stimulation and failed to bind fibrinogen, which was similar to what was previously observed with sitosterolemia mice. Thus, μ-calpain activation and FlnA degradation may partly explain the mechanism for platelet abnormalities observed in type 2B VWD.[21] Further studies are warranted to elucidate the involvement of FlnA in generation of large platelets in type 2B VWD.

Currently, type 2B VWD is treated mostly with VWF replacement therapy with adjunct therapies used for other types of VWD.[34] 1-Desamino-8-D-arginine vasopressin (DDAVP) needs to be carefully considered because of the concern for the exacerbation of thrombocytopenia. VWF A1-GPIbα has been focused as a target for the development of therapeutics against type 2B VWD.[35-37] Interestingly, MK ploidy shift, thrombocytopenia, and GPIbα cleavage were found to be corrected by administration of an antibody against VWF A1 which blocks binding to GPIbα (NMC-4). However, in agreement with our previous report on VWF^{hA1} hGPIbα mice,[13] tail bleeding time of our type 2B VWD mice was not corrected after normalization of platelet counts by high-dose NMC-4 administration. Due to the primary role in hemostasis, targeting VWF A1-GPIbα needs to be carefully studied and our animal models serve as useful tools for *in vivo* evaluation. Dysregulated VWF-GPIbα interaction can be seen not only in type 2B VWD but also in platelet-type VWD (PT-VWD), which is caused by mutations in *GP1BA*.[38] PT-VWD mouse models have been previously generated and platelet phenotypes similar to type 2B VWD have been reported.[25,39-42] Thus, results obtained from this study will

help understand other forms of hematologic disorders which involve dysregulated VWF-GPIbα interaction, and our mouse model with humanized VWF-GPIb interaction may serve as a useful tool to explore therapeutic strategy.

Disclosures

ZMR is founder, President, and CEO, AZ is Chief Innovation Officer of MERU-VasImmune, Inc. SK and TK have equity interest in MERU-VasImmune, Inc. The remaining authors declare no competing financial interests.

Contributions

SK designed and performed experiments, analyzed data, and wrote the manuscript; YM performed histologic analysis and flow cytometry analysis; HW is a director of Transgenic Core Facility, Blood Research Institute/Medical College of Wisconsin and helped to design and generate p.V1316M knockin mouse; AZ provided key reagents; RRM and ZMR supervised the study and reviewed the manuscript; TK designed, directed the study, analyzed data, and co-wrote the manuscript.

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Data-sharing statement

Further details of the data generated or analyzed in the current study are available from the corresponding author upon request.

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