FVIII-containing platelets modulate immune responses and attenuate inhibitor development in hemophilia A mice

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Supplemental Data

Supplemental Methods

Mice

Animal study procedures were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Mouse models used in this study included: 2bF8 transgenic ($2bF8^{Tg}$) mice in which human FVIII expression was driven by the platelet-specific αIIb promoter, generated by 2bF8 lentivirus-mediated oocyte transduction transgenesis,[1] and used as donors for platelet isolation. FVIII-deficient ($FVIII^{null}$, $F8^{KO}$, HA) mice with exon 17 deficiency on a mixed C57BL/6:129S [$FVIII^{null}$(B6/129)],[2] which is known to mount a stronger anti-FVIII antibody response than $FVIII^{null}$ mice in a B6 background,[3,4] or with exon 17/18 deficiency on a C57BL/6 [$F8^{KO}$(B6)] genetic background,[5] were used as recipients for platelet infusion and recombinant human FVIII (rhF8) (Xyntha, Pfizer Inc., New York) immunization. Wild-type (WT or Wt) C57BL/6 mice were used to isolate WT platelets as controls.

Platelet isolation, desialylation, and infusion

Blood sample collection and platelet isolation were performed as described in our previous report.[3] Briefly, blood samples were collected *via* retroorbital bleeds with sodium citrate as an anticoagulant. Platelets were isolated by soft spin and resuspended in Tyrode buffer. Blood

samples were collected from FVIII^{null} recipient mice one week before infusion, and platelet counts were measured using the Heska Element HT5+ Animal Blood Counter. The total blood volumes in animals were estimated based on body weights using information from the NC3Rs [National Center for Replacement, Refinement, & Reduction of Animal Research] resource library on mouse blood volume estimation (https://nc3rs.org.uk/3rs-rources). The total platelet counts in recipients were calculated based on platelet counts and estimated blood volumes. Platelets were transfused into FVIII^{null} mice up to 20-40% upon infusion, and animals were immunized with rhF8 simultaneously with a dose of 50 U/kg weekly by retro-orbital sinus injection.

Desialylated platelets (dPlts) were prepared using sialidase neuraminidase (NEU) to remove sialic acid from the ends of glycans on the GPIb protein. Various desialylation conditions were initially tested, and the optimal condition was used for the dPlt infusion studies. Under our optimal protocol, platelets (5 x 10^8/ml) were treated with 10 mU/ml of α2-3,6,8,9-neuraminidase (Millipore) (Bedford, MA) in modified Tyrode buffer, incubated at 37°C for 30 minutes (0.5 hour), then kept at room temperature for an additional 4.5 hours. After desialylation, platelets were washed and resuspended in Tyrode buffer at a concentration of 2 x 10^9 platelets/ml. To assess the percentage of desialylated platelets, 1 x 10^6 neuraminidase-treated platelets were stained with Fluorescein Ricinus Communis Agglutinin I (RCA-I) (Vector Laboratories Inc.) (Newark, CA) and analyzed via flow cytometry. JON/A antibody was used to stain activated platelet integrin α2bβ3 as described in our previous report.[6] dPlts in Tyrode buffer were infused intravenously (10 µl/g body weight) into FVIII^{null} mice to reach 20-40% upon infusion, alongside weekly rhF8 infusions for 4 weeks, or dPlt infusions alone weekly for 4 weeks, followed by rhF8 immunization. Blood samples were collected 5-7 days after dPlt and/or rhF8 infusions for immune response analysis.

Platelet lysates (2bF8^{Tg}pltLys) were prepared by freeze (-80 ℃)/thaw (37 ℃) of platelets in Tyrode buffer as reported in our previous study.[4] The amount of TGF-β1 in platelet lysates (pltTGF-β1) was measured using ELISA (Thermo Fisher Scientific) (Waltham, MA) following the procedures described in the manufacturer's instructions. pltTGF-β1 was activated by transient acidification through exposure to 1N HCl (incubate 15 minutes at room temperature), followed by neutralization with 1N NaOH to restore physiological pH, as described in the protocol

included with the ELISA kit and as previously reported.[4,7] pltTGF-β1 at a dose of 1 ng/g body weight was infused into HA mice along with rhF8 (50 U/kg) weekly *via* retro-orbital venous plexus injection for 4 weeks. Blood samples were collected one week before for platelet counts and one week after 4 doses of 2bF8^{Tg}pltLys and rhF8 infusion for immune response studies. Based on our measurement of TGF-β1 (ng/108 platelets) in 2bF8 platelet lysates determined via ELISA, as well as platelet counts and total body weight of FVIII^{null}(B6/129) recipients before infusion, the infused TGF-β1 level was converted to platelet numbers and used to estimate the corresponding percentage of platelets infused into recipients.

For co-infusion of platelets pretreated with anti-GPIb antibody experiments, platelets were isolated from 2bF8^{Tg} mice and pre-incubated with anti-GPIb monoclonal antibody (IbAb, R300) from Emfret (Eibelstadt, Germany) at a dose of 2 μg per 10^8 platelets for 40 minutes. IgG isotype (C301) was used as a control in parallel. Antibody-coated 2bF8^{Tg}Plts were infused up to 20-30% upon infusion along with rhF8 (50 U/kg) into FVIII^{null}(B6/129) mice weekly for 2 weeks, followed by two additional weeks of rhF8 infusions.

FVIII immune response studies

HA mice with or without the infusion of 2bF8^{Tg} platelets, dPlts, or platelet lysates were immunized with rhF8 (Xyntha, Pfizer) at a dose of 50 U/kg *via* intravenous (IV) injection once a week for a total of 4 weeks. Blood samples were collected from animals via retro-orbital bleeds one week after the last immunization. Platelet counts were measured by Element HT5 Veterinary Hematology Analyzer (Heska). Plasmas were isolated for assays to assess the immune response. Anti-FVIII inhibitory antibody (FVIII inhibitor) titers were determined by a modified Bethesda assay, and anti-FVIII total IgG levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the procedures described in our previous reports [8,9]. Treg cells were analyzed by flow cytometry as described in our previous report.[4,10] The T cell proliferation assay was conducted following a protocol described in our previous report[4] to evaluate CD4 T cell responses to rhF8 stimulation using *ex vivo* whole splenocyte cultures, including CD4 T cells and antigen-presenting cells (APCs), at 37ºC. Recombinant human FIX (rhF9), an unrelated antigen, served as a control in parallel. Briefly, whole splenocytes were labeled with the fluorescent dye CellTrace^{TM} Violet, and cultured in RPMI-1640 conditioned media with 2 μg/ml (10 U/ml) of rhF8 or an equal amount of unrelated antigen recombinant human FIX (rhF9, 2 μg/ml) at 37ºC

5% CO_2 for 4 days. Cell culture without rhF8 or rhF9 was used as an additional control in parallel. After culturing, cells were harvested, stained with antibodies against CD4 and TCR, and analyzed by flow cytometry to determine the percentage of daughter cells from violet-labeled CD4 T cells.

Statistical analysis

Data are presented as mean ± standard deviation (SD). One-way or two-way analysis of variance (ANOVA) or the nonparametric Friedman test was used to analyze datasets with three or more groups. A unpaired Student's *t*-test or a paired *t*-test was used to assess statistical significance between two groups. Fisher's exact test was used to assess the statistical significance of categorical variables. Statistical analysis was performed in GraphPad Prism 10. A P-value less than 0.05 was considered statistically significant.

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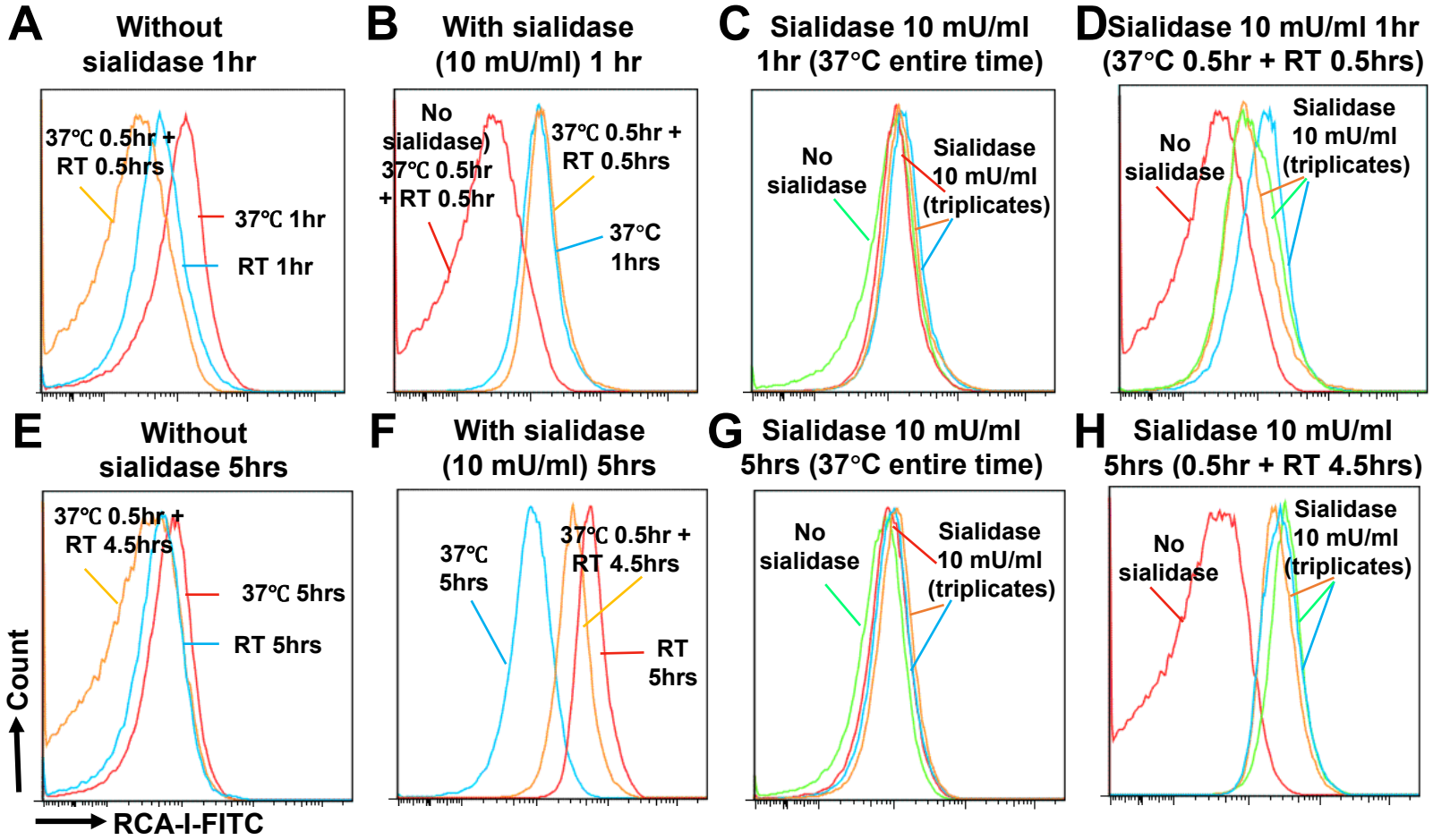
Supplemental Table 1. The abbreviations used in this study

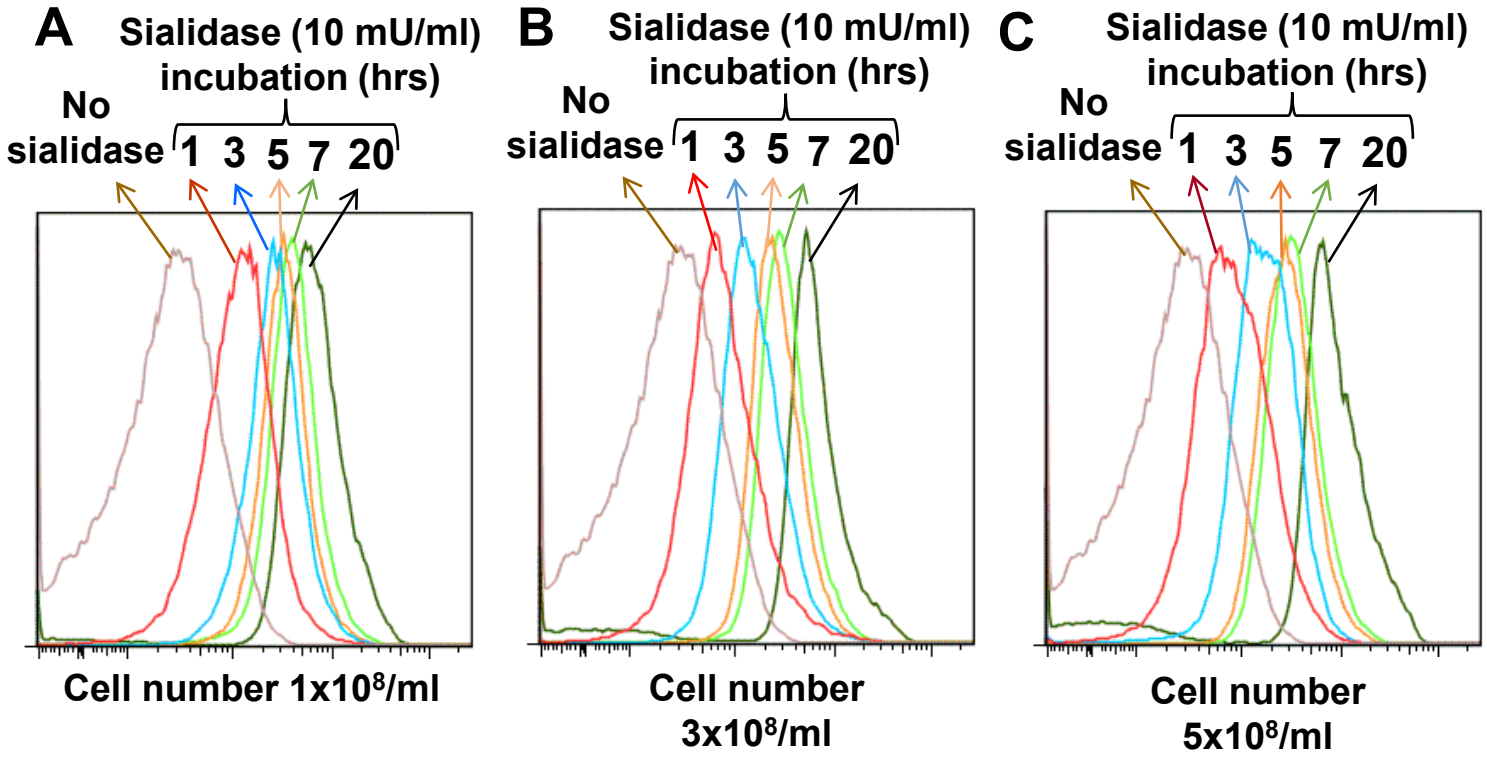
Abbreviation	Definition
HA	hemophilia A
FVIII	factor VIII
FIX	factor IX
FX	factor X
VWF	von Willebrand factor
rhF8	recombinant human FVIII
rhF9	recombinant human FIX
2bF8	human B-domain-deleted FVIII expression under the control of the platelet-specific αIIb promoter
$2bF8^{Tg}$	2bF8 transgenic mice
$2bF8^{Tg}$Plts	platelets from 2bF8 transgenic mice
dPlts	desialylated platelets
$2bF8^{Tg}$dPlts	desialylated platelets from 2bF8 transgenic mice
ITI	immune tolerance induction
TGF-β1	transforming growth factor-β1
PF4	platelet factor 4
Tregs	regulatory T cells
iTregs	induced Tregs
$FVIII^{null}$, $F8^{KO}$	FVIII-deficient or FVIII knockout
$FVIII^{null}$(B6/129)	$FVIII^{null}$ mice in a B6/129 mixed genetic background
$F8^{KO}$(B6)	FVIII knockout mice in a C57BL/6 background
WT or Wt	wild type
IV	intravenous
$2bF8^{Tg}$pltLys	platelet lysates prepared from 2bF8 transgenic mice
pltTGFβ	platelet-derived TGFβ
RT	room temperature
BU	Bethesda unit
FACS	Fluorescence-Activated Cell Sorting
ELISA	enzyme-linked immunosorbent assay
TPO-RA	thrombopoietin receptor agonist
MDSCs	myeloid-derived suppressor cells
IVIG	Intravenous Immunoglobulin

Supplemental Figure 1



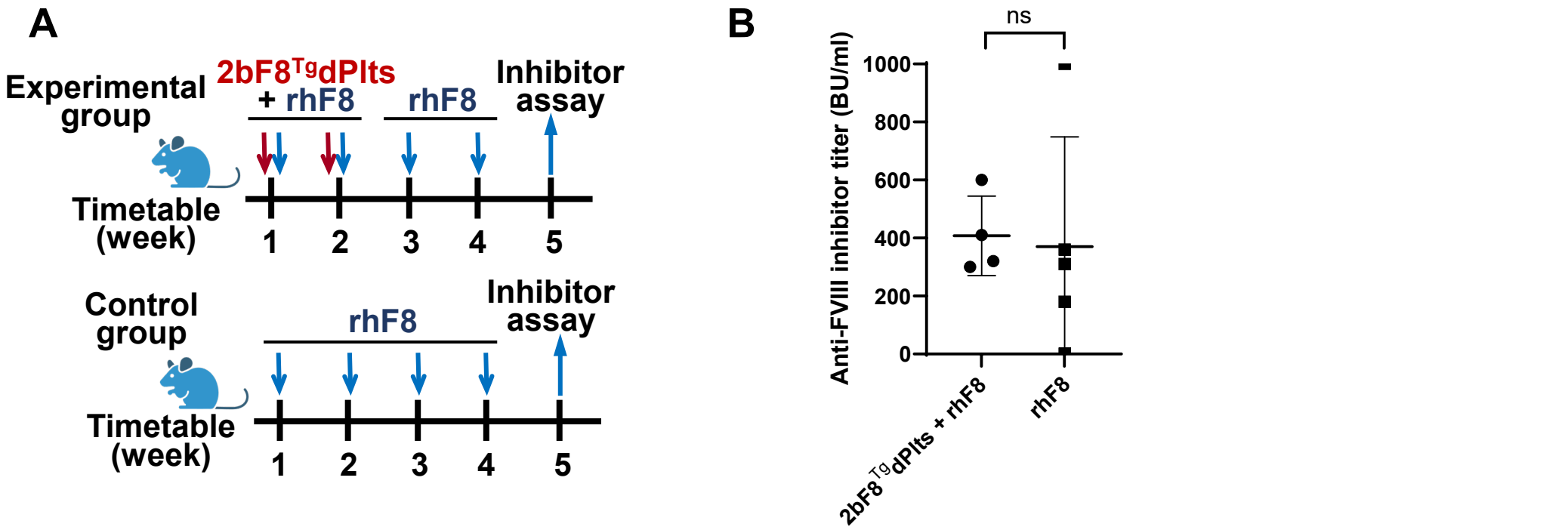
Supplemental Figure 1. Optimization of desialylating conditions to maximize platelet desialylation. Blood samples were collected from 2bF8^{Tg} mice, and platelets were isolated. Platelets (5 x 10^8/ml) were treated with 10 mU/ml of neuraminidase in modified Tyrode buffer, then incubated at various temperatures [room temperature (RT), 37°C, or both] for different durations. After incubation, the platelets were washed and resuspended in Tyrode buffer. The desialylation levels in platelets were analyzed by flow cytometry after staining with Fluorescein Ricinus Communis Agglutinin I (RCA-I) and shown in **A-H**. (**A**) Without neuraminidase for 1 hour (hr) incubation, comparing baseline at 37°C 0.5hr + RT 0.5hr, RT 1hr, vs. 37°C 1hr. (**B**) With neuraminidase for 1hr incubation, comparing 37°C 0.5hr + RT 0.5hr vs. 37°C 1hr. (**C**) With vs. without neuraminidase for 1 hour incubation at 37°C. (**D**) With vs. without neuraminidase for 0.5hr incubation at 37°C and 0.5hr at RT. (**E**) Without neuraminidase for 5 hour (hr) incubation, comparing baseline at 37°C 0.5hr + RT 4.5hr, RT 5hr, vs. 37°C 5hr. (**F**) With neuraminidase for 5hr incubation, comparing 37°C 0.5hr + RT 4.5hr, RT 5hr, vs. 37°C 5hr. (**G**) With vs. without neuraminidase for 5-hour incubation at 37°C. (**H**) With vs. without neuraminidase for 0.5hr incubation at 37°C and 4.5 hrs at RT.

Supplemental Figure 2



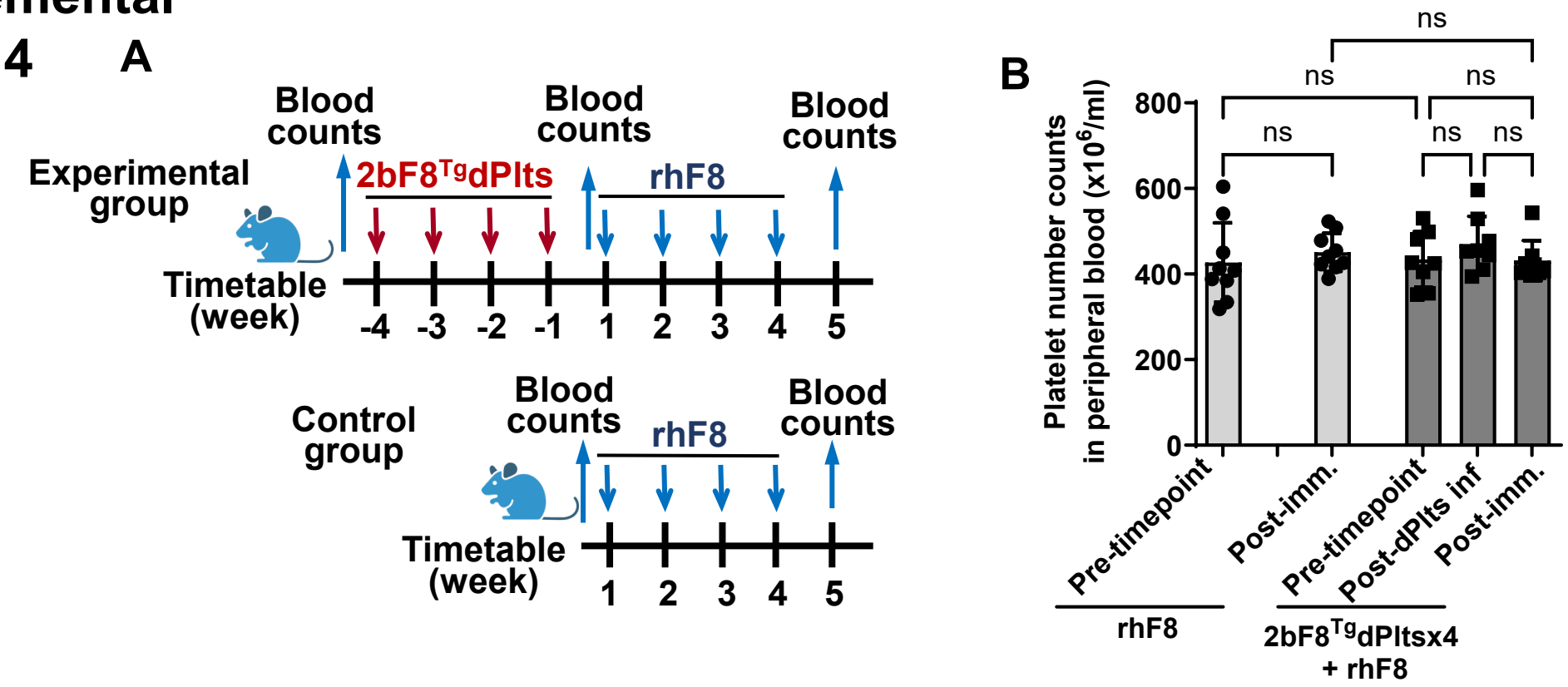
Supplemental Figure 2. Assessing the desialylation efficacy on various platelet number under our optimized desialylating conditions. Blood samples were collected from 2bF8^{Tg} mice, and platelets were isolated. Various platelet numbers (1-5 x 10^8/ml) were treated with 10 mU/ml of neuraminidase in modified Tyrode buffer, then incubated at 37°C for 0.5 hour and the remaining time at room temperature for a total incubation time of 1, 3, 5, 7, or 20 hours. After incubation, the platelets were washed and resuspended in Tyrode buffer. The desialylation levels in platelets were analyzed by flow cytometry after staining with Fluorescein Ricinus Communis Agglutinin I (RCA-I) and shown in **A-C**. (**A**) Comparison of the desialylation levels in 1 × 10^8 platelets/ml treated with 10 mU/ml of neuraminidase and incubated for various durations. (**B**) Comparison of the desialylation levels in 3 x 10^8 platelets/ml treated with 10 mU/ml of neuraminidase and incubated for various durations. (**C**) Comparison of the desialylation levels in 5 x 10^8 platelets/ml treated with 10 U/ml of Neuraminidase and incubated for various durations.

Supplemental Figure 3



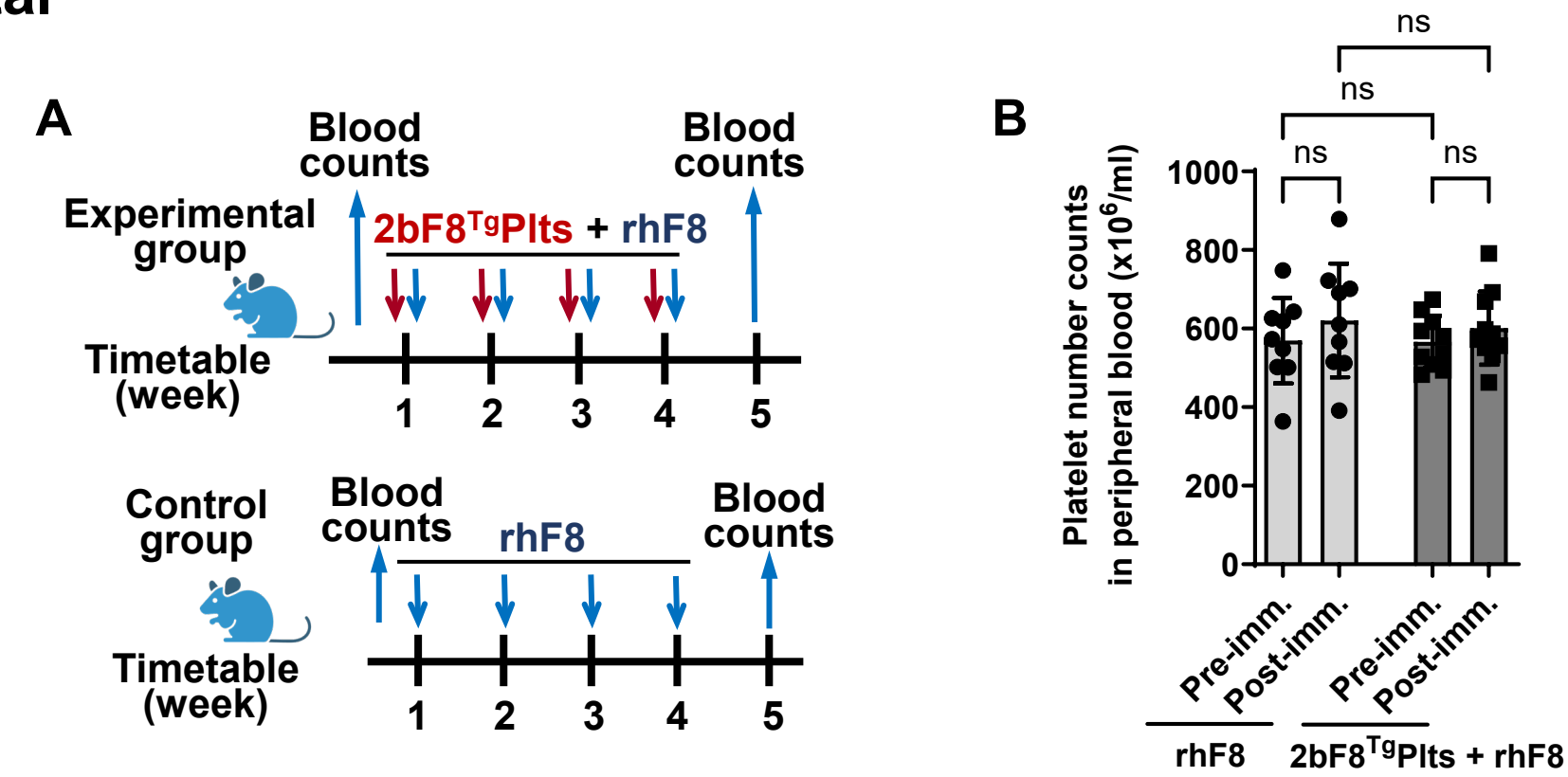
Supplemental Figure 3. The effect of co-infusion of desialylated platelets containing FVIII on FVIII immune responses in FVIII^{null}(129/B6) mice. Platelets were isolated from 2bF8 transgenic (2bF8^{Tg}) mice. 2bF8^{Tg}Plts were desialylated and infused into FVIII^{null} mice in a B6/129 mixed background [FVIII^{null}(B6/129)] along with recombinant human FVIII (rhF8) via intravenous administration weekly for 2 weeks, followed by an additional two weekly immunizations with rhF8. FVIII^{null}(B6/129) mice immunized with rhF8 weekly for 4 weeks were set up as a parallel control. One week after the last rhF8 immunization, plasma samples were collected, and FVIII inhibitor titers were measured using Bethesda assay. (**A**) Diagram of the experimental design. (**B**) FVIII inhibitor titers. “ns” indicates no statistically significant difference between the two groups by unpaired Student *t*-test.

Supplemental Figure 4



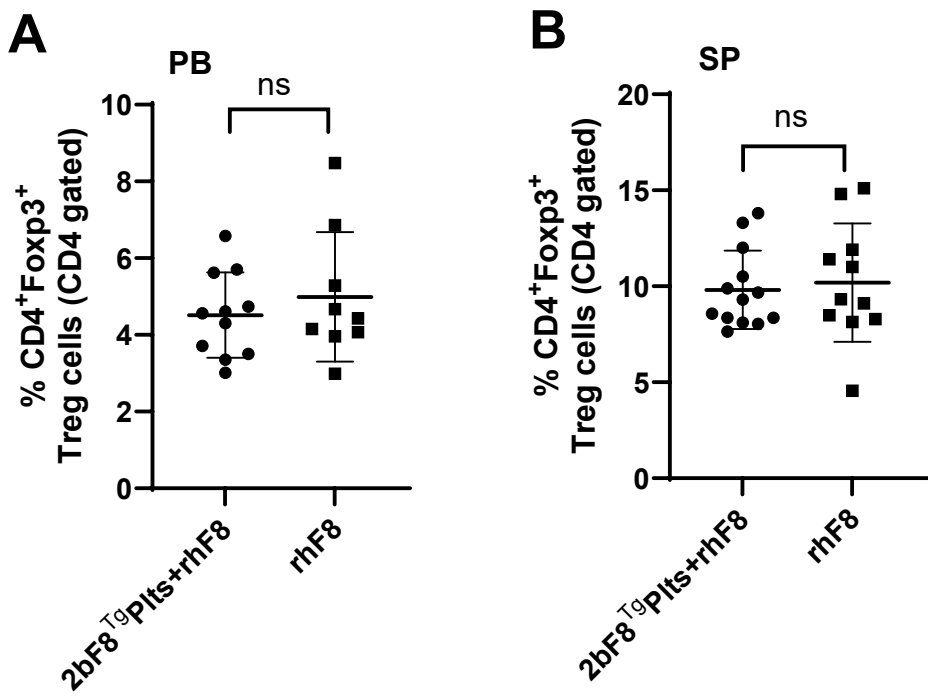
Supplemental Figure 4. The influence of transfusion of desialylated 2bF8^{Tg} platelets (2bF8^{Tg}dPlts) in FVIII^{null}(B6/129) mice. Blood samples were collected from FVIII^{null}(B6/129) mice before and after receiving 2bF8^{Tg}dPlts transfusions and after receiving recombinant human FVIII (rhF8) protein immunizations by retro-orbital bleeds. Blood samples were also collected from control-group animals that received rhF8 immunization without prior pre-transfusion of 2bF8^{Tg}dPlts. Blood counts were conducted using the Element HT5 Veterinary Hematology Analyzer (HESKA). (**A**) A schematic diagram of the timeline for blood collections is shown. (**B**) Platelet number counts are shown. “ns” indicates no statistically significant difference between the two groups by two-way ANOVA.

Supplemental Figure 5



Supplemental Figure 5. The influence of transfusion of 2bF8^{Tg} platelets (2bF8^{Tg}Plts) in F8^{KO}(B6) mice. Blood samples were collected from F8^{KO}(B6) mice before and after receiving 2bF8^{Tg}Plts & rhF8 co-infusions by retro-orbital bleeds. Blood samples were also collected from animals in the control group that received rhF8 immunization without co-infusion of 2bF8^{Tg}Plts. Blood counts were conducted using the Element HT5 Veterinary Hematology Analyzer (HESKA). (**A**) A schematic diagram of the timeline for blood collections is shown. (**B**) Platelet number counts are shown. “ns” indicates no statistically significant difference between the two groups by two-way ANOVA.

Supplemental Figure 6



Supplemental Figure 6. The effect of co-infusion of platelets containing FVIII on T regulatory (Treg) cells in F8^{KO}(B6) mice. Platelets were isolated from 2bF8 transgenic (2bF8^{Tg}) mice. 2bF8^{Tg}Plts were co-infused into FVIII^{null} mice in a B6 background [F8^{KO}(B6)] along with recombinant human FVIII (rhF8) via intravenous administration weekly for 4 weeks. F8^{KO}(B6) mice immunized with rhF8 weekly for 4 weeks were set up as a parallel control. One week after the last rhF8 immunization, blood samples and spleens were collected. Leukocytes and splenocytes were stained for CD4 and Foxp3 and analyzed by flow cytometry. (**A**) The percentages of Treg cells in the peripheral blood are shown. (**B**) The percentages of Treg cells in the spleens are shown. “PB” denotes peripheral blood. “SP” stands for the spleen. “ns” indicates no statistically significant difference between the two groups by unpaired Student *t*-test.