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

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Data-sharing statement
The authors confirm that the data supporting the findings of this study are available within the article and/or its Online Supplementary Data. For additional information, please contact the corresponding authors.

Disclosures

No conflicts of interest to disclose.

Contributions

Y.C. performed the experiments, analyzed data, and wrote the manuscript; F.X. performed the experiments and analyzed data; S.K. performed the experiments and analyzed data; J.A.S. analyzed data and edited the manuscript; W.J. discussed and interpreted part of the data; H.N. provided conceptual input on the platelet desialylation study and edited the manuscript; Q.S. designed and supervised the study, analyzed the data, and wrote the manuscript.

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Abstract

Background: Development of inhibitory antibodies (inhibitors) against FVIII is a significant complication of protein replacement therapy in hemophilia A (HA). Platelets, traditionally viewed as mediators of hemostasis, also modulate immune responses through cytokine release and interactions with immune cells. Harnessing these immunomodulatory properties may provide a novel strategy to prevent or suppress inhibitor formation. ***Objective:*** To investigate whether FVIII-engineered platelets and related platelet-based products modulate FVIII immune responses in HA mice. ***Methods:*** FVIII-containing platelets were isolated from 2bF8 transgenic mice. FVIII-deficient mice were infused with intact FVIII-containing platelets, desialylated FVIII-containing platelets (dPlts), or acidified platelet lysates in combination with or before recombinant human FVIII (rhF8) exposure. Anti-FVIII antibody titers were determined by the Bethesda assay and ELISA, and T cell responses were analyzed by flow cytometry and proliferation assays. ***Results:*** Co-infusion of FVIII platelets with rhF8 significantly reduced inhibitor titers compared with rhF8 alone. Acidified FVIII platelet lysates were potent, decreasing inhibitor titers by >20-fold when co-infused with rhF8. In contrast, co-infusion of dPlts with rhF8 did not suppress immune responses. However, repeated pre-sensitization with dPlts alone promoted immune tolerance to FVIII, evidenced by reduced inhibitor titers upon rhF8 immunization and attenuated CD4□ T cell proliferation upon subsequent rhF8 exposure. These findings reveal a hierarchy of immune modulation, with intact platelets providing partial protection, lysates strongly suppressing immune responses, and dPlts inducing immune tolerance. ***Conclusions:*** FVIII-engineered platelets and platelet-derived products are potent immune modulators. These strategies offer novel and translatable approaches to both restore hemostasis and prevent or eradicate inhibitors in HA.

Keywords: Hemophilia A; FVIII inhibitors; Platelet-targeted gene therapy; Immune tolerance.

Introduction

Hemophilia A (HA) is an X-linked bleeding disorder caused by a deficiency of factor VIII (FVIII). The standard treatment for HA is FVIII protein replacement therapy.[1] While effective, this approach is complicated by the development of inhibitory antibodies (inhibitors) against FVIII in 30–35% of patients with severe HA.[2] These inhibitors neutralize infused FVIII, making replacement therapy ineffective and presenting a clinical challenge. Immune tolerance induction (ITI), involving high-dose FVIII infusions, is the only proven method for eradicating inhibitors; however, it is lengthy, costly, and not always successful.[3]

The recent introduction of non-factor replacement therapies, such as bispecific antibody emicizumab that mimics FVIII by bridging FIXa and FX,[4] has transformed prophylactic care for HA patients with inhibitors.[5] Clinical data show significant reductions in bleeding rates with emicizumab prophylaxis.[6] However, breakthrough bleeding still occurs during trauma or surgery, indicating FVIII infusions are needed even with emicizumab.[7,8] Moreover, emicizumab cannot replace FVIII's roles in bone health and tissue remodeling,[9-11] and may cause anti-Emicizumab antibody development in some patients.[12,13] These limitations highlight the need for strategies to prevent bleeding, preserve FVIII function, and reduce immune responses that lead to inhibitor development.

Traditionally, platelets are known for their role in hemostasis, adhering to sites of injury, and releasing procoagulant factors to form a stable clot.[14] Recent studies have shown that platelets are also key modulators of immune responses.[15,16] They release cytokines, chemokines, and growth factors from α-granules, including TGF-β1 and PF4, which influence immune response pathways.[16] Platelet-derived TGF-β1 is a potent inducer of regulatory T cells (Tregs), crucial for immune regulation.[17] Aged platelets undergo apoptosis via desialylation of surface glycoproteins and are cleared by the liver and spleen, creating a tolerogenic environment that promotes Treg cell development and suppresses immune activation.[18,19] These findings suggest that platelets can uniquely deliver therapeutic proteins and regulate immune responses. Leveraging these unique properties, our group developed platelet-targeted FVIII

gene therapy using a platelet-specific promoter (αIIb) to drive FVIII (2bF8) expression, thereby storing FVIII with VWF in platelet α-granules and releasing it at the sites of injury.[20] Our studies demonstrate that platelet-targeted FVIII gene therapy corrected bleeding and induced FVIII-specific immune tolerance, even with pre-existing anti-FVIII immunity.[21-24]

In this study, we investigated whether FVIII-engineered platelets could actively modulate FVIII immune responses and prevent inhibitor formation. We focused on three approaches in FVIII-deficient mice: (1) co-infusion of FVIII-engineered platelets with recombinant human FVIII (rhF8) to test their effect during initial FVIII exposure, (2) administration of acidified FVIII platelet lysates to evaluate the role of platelet-derived regulatory factors, and (3) infusion of desialylated FVIII-engineered platelets to direct clearance through tolerogenic pathways. Our findings demonstrate that FVIII-engineered platelets can suppress inhibitor development and promote tolerance through Treg-mediated mechanisms, providing a foundation for a novel therapeutic strategy to address both the hemostatic and immunologic challenges of HA.

Methods

Detailed methods and statistics used in this study are in the Supplemental Data. Abbreviations used are listed in **Supplemental Table 1**.

Mice

Animal studies were approved by the IACUC at the Medical College of Wisconsin. Mouse models in this study included 2bF8 transgenic ($2bF8^{Tg}$) mice, which expressed human FVIII driven by the platelet-specific αIIb promoter (2bF8)[25] 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)][26] or with exon 17/18 deficiency on a C57BL/6 [$F8^{KO}$(B6)] genetic background,[27] were used as recipients for platelet infusion and rhF8 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 reported.[28] Isolated platelets were transfused into $FVIII^{null}$ mice, and animals were immunized with rhF8.

Desialylated platelets (dPlts) were prepared using neuraminidase, washed, and resuspended in Tyrode buffer at a concentration of $2x10^9$ platelets/ml. To determine the percentage of desialylated platelets, neuraminidase-treated platelets were stained with RCA-I and analyzed by FACS. JON/A antibody was used to stain activated platelet integrin α2bβ3 as reported.[29] dPlts were infused intravenously (IV) into $FVIII^{null}$ mice to reach 20-40% with weekly rhF8 for 4 weeks, or dPlt alone weekly for 4 weeks, then rhF8 immunization. Blood samples were collected 5-7 days after infusion for immune response studies.

Platelet lysates from $2bF8^{Tg}$ mice ($2bF8^{Tg}$pltLys) were prepared by freezing and thawing platelets, as previously reported.[30] The amount of TGF-β1 in platelet lysates (pltTGF-β1) was measured using ELISA. pltTGF-β1 was activated by transient acidification.[30,31] pltTGF-β1 (1ng/g) was infused into HA mice along with rhF8 weekly for 4 weeks.

For co-infusion of platelets pretreated with anti-GPIb antibody experiments, platelets isolated from $2bF8^{Tg}$ mice were pre-incubated with anti-GPIb antibody (R300, Emfret) at a dose of $2μg/10^8$ platelets. IgG isotype (C301) was used as a control in parallel. Antibody-coated $2bF8^{Tg}$Plts were infused along with rhF8 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 $2bF8^{Tg}$Plts, dPlts, or pltLys were administered with rhF8 at a dose of 50 U/kg/week by IV injection for 4 weeks. Blood samples were collected from animals one week after the last immunization. Platelet counts were measured by a Heska Element HT5 Analyzer. Plasmas were isolated for the Bethesda assay and ELISA to measure FVIII inhibitor titers and anti-FVIII IgG levels, as previously reported.[20,22] Treg cells

were analyzed by FACS.[30,32] T cell proliferation assay[30] was conducted to evaluate CD4 T cell responses to rhF8 stimulation.

Results

Co-infusion of platelets containing FVIII together with rhF8 immunization suppressed FVIII immune responses

Our previous study shows that infusion of FVIII-engineered ($2bF8^{Tg}$) platelets alone neither elicits anti-FVIII immune responses nor induces immune tolerance in $FVIII^{null}$(B6/129) mice.[25] To investigate how co-infusing $2bF8^{Tg}$ platelets with rhF8 affects FVIII immune responses, we transfused $2bF8^{Tg}$ platelets and rhF8 into $FVIII^{null}$(B6/129) mice, which mount a stronger FVIII response than on a B6 background,[28,30] *via* weekly IV injections for two weeks, followed by two more rhF8 doses, as shown in **Figure 1A**. One week after the last rhF8 infusion, blood samples were collected for analysis of anti-FVIII antibody development. As shown in **Figure 1B**, the inhibitor titers in the $2bF8^{Tg}$ platelets plus rhF8 co-infused [$2bF8^{Tg}$Plts + rhF8] group were 7.9-fold lower than those in the rhF8 infusion alone [rhF8] group (32.02±35.81 BU/ml and 253.33±125.32 BU/ml, respectively, $P<0.01$). The anti-FVIII total IgG in the $2bF8^{Tg}$Plts and rhF8 co-infusion group was also significantly lower than in the rhF8 group (320±196 versus 2720±2081) (**Figure 1C**). These results demonstrated that co-infusion of platelets containing FVIII with rhF8 does not increase anti-FVIII immune responses; instead, it mitigates the development of FVIII antibodies in HA mice.

Platelet lysate-mediated immune suppression and biological effects of anti-GPIb–coated platelets

TGF-β1 is a crucial regulator of the immune system that maintains immune homeostasis. Our previous study showed that 97% of circulating TGF-β1 is stored in platelets.[30] Acidified TGF-β1 from platelet lysates (pltLys) effectively induces Treg cells (iTregs), which suppress FVIII immune responses better than purified TGF-β1-induced Tregs in HA mice.[30] Here, we investigated whether co-infusion of $2bF8^{Tg}$pltLys with rhF8 influences FVIII inhibitor development. In our preliminary study, we co-infused unacidified $2bF8^{Tg}$pltLys with rhF8

and found it did not mitigate FVIII inhibitor development. Thus, we acidified 2bF8^{Tg}pltLys to activate latent TGF-β1 to an immunoreactive form as previously reported.[30,31] The acidified 2bF8^{Tg}pltLys were co-infused with rhF8 into FVIII^{null}(B6/129) mice weekly for 4 weeks (**Figure 2A**). The amount of pltTGF-β1 infused into recipients was estimated to be comparable to that from approximately 15-20% of the infused platelets. As shown in **Figure 2B**, the FVIII inhibitor titers in FVIII^{null}(B6/129) mice that received rhF8 immunization with co-infusion of 2bF8^{Tg}pltLys were significantly lower compared to those in animals without co-infusion (23.33±25.17 BU/ml versus 506.7±257.2 BU/ml). Platelet counts were similar in animals before and after four co-infusions of 2bF8^{Tg}PltLys and rhF8 (**Figure 2C**).

Our previous study showed that *in vivo* platelet deletion using anti-GPIb antibody (IbAb) (R300) infusion could induce Treg cells and suppress FVIII immune responses in FVIII^{null}(B6/129) mice.[30] We wondered whether we could prepare platelets coupled with IbAb *in vitro*, then transfuse them to mimic *in vivo* platelet deletion and modulate FVIII immune responses. We tested whether co-infusing 2bF8^{Tg}Plts preincubated with R300, along with rhF8 infusions, affects FVIII immune responses. We incubated isolated 2bF8^{Tg}Plts with R300 (2μg/10^8 platelets) to simulate an *in vivo* dose of 1mg/kg of anti-GPIb antibody. IgG isotype (C301) served as a control (**Figure 2D**). As shown in **Figure 2E**, there was no significant difference in FVIII inhibitor titers developed in animals that received co-infusion of anti-GPIb antibody-coated 2bF8^{Tg}Plts [2bF8^{Tg}Plts(R300)] versus IgG isotype-treated 2bF8^{Tg}Plts(C301). Interestingly, platelet counts were significantly elevated after infusions of R300- or C301-opsonized platelets (**Figure 2F**).

These results demonstrate that acidified 2bF8^{Tg}pltLys can attenuate FVIII inhibitor development, consistent with activation of platelet-derived TGF-β1, whereas infusion of anti-GPIb-coated 2bF8^{Tg}Plts did not affect inhibitor titers but was associated with increased peripheral platelet counts.

Infusion of desialylated 2bF8^{Tg}Plts induces immune tolerance in FVIII^{null} mice

It is known that apoptosis plays a crucial role in immune tolerance,[33-35] and targeting antigens to apoptotic cells can lead to immune tolerance to the antigens of interest.[36-38] Aged platelets undergo apoptosis while their glycan proteins are desialylated,[19] creating an immunomodulatory environment *in vivo*.[18] Here, we evaluate the impact of desialylated platelets that contain FVIII (2bF8^{Tg}dPlts) on FVIII immune responses in HA mice. First, we optimized our platelet desialylation protocol to ensure that greater than 95% of platelets were desialylated while remaining inactivated. We tested various concentrations of sialidase (neuraminidase) and platelet numbers under different incubation conditions. Interestingly, we found that incubating platelets at 37ºC for more than 1 hour induced some degree of desialylation, and neuraminidase became ineffective (**Supplemental Figure 1A, C, E, and G**). Incubating at room temperature (RT) also caused desialylation, although to a lesser extent than at 37°C, and adding neuraminidase could further desialylate platelets (**Supplemental Figure 1A, B, E, and F**). Incubating at 37ºC for 0.5 hour, followed by RT incubation for an additional 4.5 hours, could maximize sialidase activity (**Supplemental Figure 1D and H and Figure 3A-D**) while still protecting platelets from activation (**Figure 3E**). With 10mU/ml of neuraminidase following our optimized incubation condition, a cell number between $1x10^8$-$5x10^8$ platelets/ml could be effectively desialylated (**Supplemental Figure 2**).

Using our optimal protocol, we evaluated how 2bF8^{Tg}dPlts infusion affects FVIII immune responses in HA mice. We first co-infused 2bF8^{Tg}dPlts together with rhF8 weekly into FVIII^{null}(B6/129) mice for two weeks, followed by an additional two weeks of rhF8 immunizations. We found no significant difference in FVIII inhibitor titers in mice that received 2bF8^{Tg}dPlts co-infusion versus the rhF8 groups (**Supplemental Figure 3**). We wondered whether it takes time to sensitize the immune system with 2bF8^{Tg}dPlts before exposing it to rhF8. We infused 2bF8^{Tg}dPlts alone, without rhF8, weekly for 4-5 weeks as an alternative platelet-based immune modulation strategy, then challenged with rhF8 weekly for 4 weeks (**Figure 4A**). We found that platelet counts were comparable in animals before and after 2bF8^{Tg}dPlts infusions (**Supplemental Figure 4**).

There were no detectable FVIII inhibitors in HA mice after receiving 4 or 5 rounds of $2bF8^{Tg}$dPlts infusions. After rhF8 immunizations, the FVIII inhibitor titers in $FVIII^{null}$(B6/129) mice that received $2bF8^{Tg}$dPlt transfusions, including both 4 and 5 infusions, were significantly lower than in those without $2bF8^{Tg}$dPlt transfusion (100.3±142.71 BU/ml vs. 351.16±211.34 BU/ml, respectively), but there was no significant difference between the groups after 4 and 5 sensitization groups (**Figure 4B and C**). Following rhF8 immunization, the incidence of high FVIII inhibitors (greater than 25 BU/ml) in the group pre-sensitized with $2bF8^{Tg}$dPlts was significantly lower than in the group without pre-sensitization (**Figure 4D**). There was no significant difference in anti-FVIII total IgG titers between the two groups (**Figure 4E**).

To investigate the potential mechanism by which the immune response was modulated by $2bF8^{Tg}$dPlt transfusions, we monitored Tregs by FACS. We found that the frequency of Tregs in peripheral blood significantly increased in $FVIII^{null}$ mice after $2bF8^{Tg}$dPlts transfusions but remained comparable after rhF8 immunization (**Figure 5A and B**). There was no significant difference in outcomes after rhF8 immunization between the groups that received or did not receive $2bF8^{Tg}$dPlts pre-sensitizations (**Figure 5C**). We further performed the T cell proliferation assay[30] on whole splenocytes isolated from mice one week after the last rhF8 immunization. As shown in **Figure 5D and E**, the daughter $CD4^+$ T cells from splenocytes that were pre-sensitized with $2bF8^{Tg}$dPlts did not significantly increase upon rhF8 stimulation (1.38±0.59-fold) compared to conditions without rhF8 or unrelated rhF9 treatments. In contrast, after rhF8 stimulation, the daughter CD4 T cells from the control group without $2bF8^{Tg}$dPlt sensitization increased 4.96±1.27-fold compared to the condition without rhF8 treatment.

Taken together, these results demonstrate that the infusion of $2bF8^{Tg}$dPlts does not elicit FVIII-specific immune responses but induces Treg cell expansion. Pre-sensitization with $2bF8^{Tg}$dPlts can attenuate immune responses to FVIII in $FVIII^{null}$ mice.

The immune modulation of 2bF8^{Tg}Plt transfusion in FVIII^{null} mice in a C57BL/6 genetic background

We further assessed the impact of 2bF8^{Tg}Plt transfusion on anti-FVIII immune responses in F8^{KO}(B6) mice, which we previously developed by deleting exons 17 and 18 of the murine *F8* gene.[27] This line of animals was well suited to platelet-rhF8 co-transfusion studies, as all animals survived the full four doses of 2bF8^{Tg}Plts and rhF8 co-infusions. In comparison, FVIII^{null}(B6/129) mice died after 3-4 doses of 2bF8^{Tg}Plts and rhF8 co-infusions, although they all survived with more than four doses of a single infusion of either 2bF8^{Tg}Plts or rhF8.

In the F8^{KO}(B6) colony, after four doses of rhF8 immunization at 50 U/kg/week, 93% (n=14) mice developed FVIII inhibitors with a titer of 27.66±23.59 BU/ml, ranging from 2.3-90 BU/ml. The FVIII inhibitor titers in F8^{KO}(B6) mice were significantly lower than those in FVIII^{null}(B6/129) mice following the same immunization protocol, confirming that the genetic background affects FVIII inhibitor development. In contrast, 57% (n=14) of animals did not develop detectable FVIII inhibitors after immunization with rhF8 and co-infusion with 2bF8^{Tg}Plts. The inhibitor titer in the 2bF8^{Tg}Plts and rhF8 co-infused [2bF8^{Tg}Plts + rhF8] group was 17.5±28.6 BU/ml, which was significantly lower than in the rhF8 group **(Figure 6A-C**). The platelet number counts were comparable in animals before and after four doses of 2bF8^{Tg}Plts and rhF8 co-infusions (**Supplemental Figure 5**). The frequency of Treg cells in the peripheral blood and spleen in the 2bF8^{Tg}Plts and rhF8 co-infused group was comparable to the rhF8 group (**Supplemental Figure 6**).

No FVIII inhibitors were detected in animals that received 2bF8^{Tg}Plts transfusion (**Figure 6C**). The titer of anti-FVIII total IgG in the 2bF8^{Tg}Plts and rhF8 co-infused group was also significantly lower than in the rhF8 group (**Figure 6D**). We compared the animals immunized with rhF8 and co-infused with wild-type platelets (WtPlts), observing no significant difference in the incidence of FVIII inhibitor development or the titer of FVIII inhibitors between the rhF8 group and the rhF8 and WtPlt co-infused group (**Figure 6B-D**). Finally, we assessed the immunomodulatory efficacy of desialylated 2bF8^{Tg}Plts (2bF8^{Tg}dPlts) on FVIII

immune responses by pre-sensitizing $F8^{KO}$(B6) mice, following a protocol similar to that used for $FVIII^{null}$(B6/129), as depicted in **Figure 4A**. When $F8^{KO}$(B6) mice were pre-sensitized with $2bF8^{Tg}$dPlts weekly for 4 weeks, followed by rhF8 immunization, the incidence of inhibitor development, the titers of FVIII inhibitors, and anti-FVIII total IgG in the $2bF8^{Tg}$dPlts pre-sensitized group were significantly lower than those in the rhF8 group (**Figure 6B-D**)

Taken together, these results confirm that the co-infusion of platelets containing FVIII can suppress anti-FVIII immune responses, and pre-sensitized HA mice treated with desialylated platelets containing FVIII can modulate FVIII antibody development.

Discussion

In this study, we demonstrated that FVIII-engineered platelets can actively modulate FVIII immune responses. Our data reveal that multiple platelet-based methods, including co-infusion of FVIII-containing platelets with rhF8, infusion of acidified platelet lysates, and transfusion of desialylated platelets containing FVIII, can significantly reduce or even prevent FVIII inhibitor development in HA mice. These results reveal that FVIII-engineered platelets serve dual roles: delivering FVIII to restore hemostasis and modulating immune tolerance for HA with FVIII protein infusion.

Previous studies show platelets can deliver FVIII directly to injury sites when needed, bypassing inhibitor inactivation.[20,21,28] Our current study suggests that the infusion of FVIII-engineered platelets also contributes to immune regulation. One notable observation in our study was that HA mice co-infused with FVIII-containing platelets and rhF8 exhibited significantly lower FVIII inhibitor titers than those receiving rhF8 alone. This suggests that platelet-delivered FVIII, along with its platelet contents, has an immunomodulatory effect during antigen exposure to rhF8. The exact mechanism by which co-infusion of FVIII-engineered platelets modulates FVIII immune responses remains unclear. The attenuated immune response is more plausibly explained by antigen delivery in a platelet-derived, TGF-

β1-rich tolerogenic milieu rather than by physical sequestration of rhF8. Platelets can directly interact with dendritic cells,[39,40] monocytes,[41] and lymphocytes via surface molecules such as P-selectin and integrins.[42,43] These interactions may skew immune responses by delivering inhibitory signals or facilitating antigen transfer under non-inflammatory conditions. FVIII-engineered platelets may directly engage splenic antigen-presenting cells (APCs) or marginal zone macrophages, routing FVIII to tolerogenic pathways and away from the strongly immunogenic presentation that occurs when free rhF8 is internalized alone. The finding that co-infusion lowers FVIII inhibitor titers has significant translational implications, as patients could potentially receive both FVIII-engineered platelets, if available in the future, and rhF8 to enhance hemostatic effectiveness, when needed, such as during surgery, while reducing immunogenicity. Further studies are warranted to explore the potential mechanisms underlying the immunomodulatory function of co-infusion of FVIII-engineered platelets and rhF8.

It has been demonstrated that TGFβ is a significant immunomodulatory component in FVIII immune responses.[44-47] Peng and colleagues demonstrated that TGFβ levels increased in HA mice after infusion of an anti-CD3 antibody to deplete T cells, leading to immune tolerance to FVIII.[45] A study by Kallas et al. demonstrated that adding recombinant latent TGFβ to an FVIII infusion significantly decreased the antibody response to FVIII compared with FVIII treatment alone, suggesting that TGFβ has a protective, immunosuppressive effect on FVIII immune responses at least in mice.[47] Our findings that acidified platelet lysates (containing TGF-β1) suppress FVIII inhibitor development align with the role of activated pltTGF-β1 in promoting immune regulation. Recent studies in chronic ITP show that TPO-RA treatment increases platelet mass, enhances pltTGF-β1 interactions, and reprograms MDSCs via TGF-β/Smad signaling, thereby aiding immune regulatory homeostasis.[48] These suggest that pltTGF-β1 may represent a shared immunoregulatory axis across different immune-mediated hematologic disorders, although engaged through distinct activation pathways.

In our current study, we found that co-infusion of acidified, but not unacidified, lysates

prepared from 2bF8^{Tg} platelets significantly reduced the development of FVIII inhibitors. This aligns with our earlier report, which showed that platelet lysates are rich in TGF-β1 and other immunomodulatory molecules that effectively induce Foxp3 Treg cells.[30] Platelet lysate-induced Tregs have been previously shown to exhibit superior stability and suppressive capacity compared to iTregs generated using purified TGF-β1 alone, resulting in a significant reduction in FVIII immune responses *in vivo*.[30] The current study extends these observations by demonstrating that platelet lysates containing FVIII not only induce Treg differentiation *in vitro* [30] but also directly suppress the generation of FVIII inhibitors during concurrent rhF8 immunization. These results reveal the dual role of platelet contents: creating a tolerogenic cytokine environment and presenting the FVIII antigen noninflammatory, thereby promoting antigen-specific tolerance. Platelet lysates could serve as a cell-free immune therapy, avoiding the complexities of live platelet transfusion while leveraging their immunoregulatory effects. Whether metabolic modulators, such as D-mannose, which boost TGF-β1-dependent tolerance in other immune-mediated hematologic models,[49] can further enhance platelet-derived immune regulation in HA remains to be explored.

Our prior work showed that *in vivo* administration of anti-GPIb antibodies during rhF8 infusion causes significant platelet depletion, promotes Treg cell expansion, and reduces FVIII inhibitor development.[30] In this study, pre-incubation of 2bF8 platelets with anti-GPIb or IgG, followed by co-infusion with rhF8, did not affect inhibitor titers. This discrepancy might reflect the extent of platelet clearance: systemic anti-GPIb antibodies target all platelets and create a tolerogenic environment, whereas opsonized 2bF8^{Tg}Plts constitute only a small fraction and are cleared gradually *in vivo*. Under these conditions, the tolerogenic signal from a small number of antibody-coated platelets is probably insufficient to counteract the strong immunogenic stimulus from soluble rhF8. Interestingly, repeated co-infusion of rhF8 with 2bF8^{Tg}Plts pre-incubated with anti-GPIb or isotype IgG led to increased peripheral platelet counts after four weekly infusions. A possible explanation is that IgG-opsonized platelets are cleared via Fcγ receptor pathways, and repeated dosing saturates these receptors on macrophages, mimicking the effects of low-dose IVIG or immune complexes. This reduces

endogenous platelet clearance and increases thrombopoiesis, thereby raising platelet counts. Conversely, 2bF8^{Tg}Plts without antibody do not activate Fcγ receptors and do not alter platelet homeostasis.

Our previous study has demonstrated that infusion of intact platelets containing FVIII cannot induce immune tolerance in HA mice, whereas preconditioning animals with sublethal irradiation followed by 2bF8^{Tg}Plts infusion can, an effect attributed to resynchronizing the immune system.[25] In the current study, we demonstrated that repeated transfusion of desialylated FVIII-engineered platelets (2bF8^{Tg}dPlts) induced immune tolerance. Mice pre-sensitized with 2bF8^{Tg}dPlts showed reduced inhibitor titers and reduced CD4□ T cell proliferation upon subsequent rhF8 exposure. This suggests that desialylation triggers tolerogenic clearance pathways, consistent with reports that desialylated platelets are rapidly removed by hepatocytes and splenic macrophages, creating an immunosuppressive environment.[18,19] Moreover, infusion of 2bF8^{Tg}dPlts significantly increased circulating Tregs, providing a potential mechanistic link between desialylated platelet clearance and tolerance induction. This aligns with studies showing that apoptotic cells can drive tolerance by presenting antigens in a non-inflammatory context.[33,50] Our results suggest that 2bF8^{Tg}dPlts act as tolerogenic vehicles, delivering FVIII antigen to APCs in a manner analogous to apoptotic cell therapy. Importantly, our optimized desialylation protocol achieved greater than 95% efficiency without platelet activation, ensuring that the observed effects were due to glycan modification rather than platelet activation artifacts.

Our findings collectively reveal a hierarchy of immune modulation: co-infusion of 2bF8^{Tg}Plts with rhF8 for half of the course reduced but did not fully prevent inhibitor development in a mixed genetic background, indicating a partial protective effect. However, it could completely abolish FVIII immune responses in HA mice with a C57BL/6 genetic background when each rhF8 infusion was paired with co-infusion of 2bF8^{Tg}Plts. Acidified platelet lysates elicited more pronounced suppression, likely via direct TGF-β1-mediated immunomodulation. Repeated presensitization with 2bF8^{Tg}dPlts induced immune tolerance, potentially leading to

FVIII unresponsiveness. These complementary strategies offer flexibility for different clinical scenarios. For example, co-infusion of FVIII platelets with rhF8 could be used prophylactically at the time of initial FVIII exposure in previously untreated patients to lower the risk of inhibitor formation. Platelet lysates could be used as an adjunct to ITI protocols or in patients in whom live platelet transfusion is not feasible. dPlt therapy may provide a means to induce tolerance before or after inhibitor development, potentially replacing or enhancing current ITI regimens. Given that platelet transfusion is already a routine and safe clinical practice, these approaches could be rapidly translated into clinical testing.

Our current study has some limitations, and several questions remain unanswered. First, although we demonstrated Treg cell expansion and reduced effector T cell proliferation, further research is necessary to elucidate the cellular and molecular pathways by which platelets and their derivatives induce tolerogenic effects. Second, the durability of tolerance remains uncertain. In follow-up studies (data not shown), weekly rhF8 exposure eventually elicited FVIII inhibitors, indicating that tolerance induced by dPlts is not permanent and can be overridden by ongoing antigenic stimulation. This suggests multiple or periodic doses may be necessary to maintain tolerance, with future studies needed to optimize dosing strategies. Lastly, it will be important to explore how these strategies might interact with existing non-factor treatments, such as emicizumab. Combining these approaches could provide synergistic benefits, including ongoing bleed protection, prevention of inhibitor development, and maintenance of FVIII’s extra-hemostatic functions.

In conclusion, this study provides compelling evidence that FVIII-engineered platelets and platelet-derived products are not only hemostatic agents but also potent immune modulators. By leveraging platelets' natural biology, these strategies offer a novel approach to simultaneously correcting bleeding and preventing inhibitor formation in HA. These findings lay the groundwork for innovative interventions to address one of the most significant challenges in hemophilia care: the development of FVIII inhibitors.

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Figure legends

Figure 1. The modulatory effect of platelets containing FVIII on FVIII immune responses in FVIII^{null}(B6/129) mice. Platelets were isolated from 2bF8 transgenic (2bF8^{Tg}) mice. 2bF8^{Tg}Plts were infused into FVIII^{null} mice in a B6/S129 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. Anti-FVIII total IgG levels were determined by ELISA. (**A**) Diagram of the experimental design. (**B**) FVIII inhibitor titers. (**C**) Anti-FVIII total IgG titers. Data are summarized from 2 trials of 2bF8^{Tg}Plts infusion studies. *P < 0.05; **P < 0.01 by the unpaired Student t-test.

Figure 2. The modulatory effect of lysates from 2bF8^{Tg} platelets or anti-GPIb antibody-coated 2bF8^{Tg} platelets on FVIII immune responses in FVIII^{null}(129/B6) mice. Blood samples were collected from 2bF8 transgenic (2bF8^{Tg}) mice, and platelets were isolated. Platelet lysates (2bF8^{Tg}pltLys) were prepared by the freeze/thaw method, and TGF-β1 levels in the lysates were measured using an enzyme-linked immunosorbent assay. TGF-β1 was activated through HCl acidification followed by NaOH neutralization. An acidified platelet lysate containing 1 ng/g of TGF-β1 was co-infused with rhF8 (50 U/kg) into FVIII^{null}(129/B6) mice via intravenous injection weekly for 4 weeks. Animals that received only rhF8 immunizations were used as a control in parallel. For anti-GPIb antibody (R300)-coated platelet infusion, 2bF8^{Tg} platelets were pre-incubated with either anti-GPIb antibody or isotype control (C301) at a concentration of 2 μg per 10^8 platelets for 40 minutes. Antibody-incubated platelets were co-infused with rhF8 (50 U/kg) weekly for 4 weeks. One week after the last infusion, plasma samples were collected, and FVIII inhibitor titers were determined by Bethesda assay. (**A**) Diagram of the experimental design for the 2bF8^{Tg}pltLys infusion study. (**B**) FVIII inhibitors in the 2bF8^{Tg}pltLys study. (**C**) Platelet counts in mice before and

after 4 doses of rhF8, with or without co-infusion of 2bF8^{Tg}pltLys. (**D**) Diagram of the experimental design for the anti-GPIb antibody-coated 2bF8^{Tg}Plts infusion study. (**E**) FVIII inhibitor titers in the anti-GPIb antibody-coated 2bF8^{Tg}Plts infusion study. (**F**) Platelet counts from mice before and after 4 doses of rhF8, with co-infusion of anti-GPIb antibody (R300) or isotype IgG control (C301) opsonized 2bF8^{Tg}Plts. (**B-C**) data are summarized from 2 trials of 2bF8^{Tg}pltLys infusion studies. (**E-F**) data are summarized from 2 trials of GPIb antibody opsonized 2bF8^{Tg}Plts infusion studies. *P < 0.05; "ns" indicates no statistically significant difference between the two groups by the unpaired Student *t*-test (**B and E**) or two-way ANOVA (**C** and **F**).

Figure 3. Optimization of desialylating conditions to maximize platelet desialylation while minimizing platelet activation. 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 α2-3,6,8,9-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). Platelet activation levels were assessed by JON/A antibody staining and flow cytometry. (**A**) Baseline desialylation levels in 2bF8^{Tg}Plts without added neuraminidase, incubated at 37°C for 0.5 hour, followed by further incubation at room temperature. (**B**) Desialylation levels in 2bF8^{Tg}Plts with 10 mU/ml Neuraminidase, incubated at 37°C for 0.5 hour, followed by further incubation at room temperature. The condition without adding sialidase, but incubating at 37°C for 0.5 hours, followed by incubation at room temperature for a total of 1 hour, served as the baseline control. (**C**) Baseline desialylation levels in 2bF8^{Tg}Plts without added neuraminidase, incubated at room temperature for varying durations of hours. (**D**) Desialylation levels in 2bF8^{Tg}Plts with 10 mU/ml Neuraminidase, incubated at room temperature for varying durations of hours. The condition without adding sialidase but incubating at room temperature for 1 hour was used as a baseline control. (**E**) Activation levels in platelets after incubating with or without Neuraminidase under varying incubation

conditions. Platelets were stained with JON-A antibody, which labels activated αIIbβ3, and analyzed by flow cytometry.

Figure 4. The immune tolerance induction effect of desialylated platelets containing FVIII on FVIII immune responses in FVIII^{null} (B6/129) mice. Blood samples were collected from 2bF8^{Tg} mice, and platelets were isolated. These platelets were desialylated using our optimized protocol (10 mU/ml of Neuraminidase with 5x10^{8} platelets/ml, incubated at 37°C for 0.5 hours, then at room temperature for 4.5 hours). The desialylated platelets (2bF8^{Tg}dPlts) were infused into FVIII^{null} mice on a B6/S129 mixed background [FVIII^{null} (B6/129)] weekly for 4-5 weeks, followed by immunization with recombinant human FVIII (rhF8) *via* intravenous injection at 50 U/kg/week for 4 weeks. FVIII^{null} (B6/129) mice immunized with rhF8 weekly for 4 weeks served as a control group. One week after the last infusion of 2bF8^{Tg}dPlts and the final rhF8 immunization, plasma samples were collected, and FVIII inhibitor titers and anti-FVIII total IgG levels were measured. (**A**) Diagram of the experimental design. (**B**-**C**) FVIII inhibitor titers. (**D**) Data in the incidence of animals that developed FVIII inhibitor titers greater than 25 BU/ml. The 2bF8^{Tg}dPlts + rhF8 group presented in (**C** and **D**) included data from mice with 4x and 5x dPlts pre-sensitizations. (**E**) Anti-FVIII total IgG titers. The 2bF8^{Tg}dPlts + rhF8 group presented in (**E**) included data from mice with 4x dPlts pre-sensitizations. Data are summarized from 4 trials of 2bF8^{Tg}dPlts infusion studies $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; "ns" indicates no statistically significant difference between the two groups by either one-way ANOVA (**B**), unpaired Student *t*-test (**C** and **E**), or Fisher's exact test (**D**).

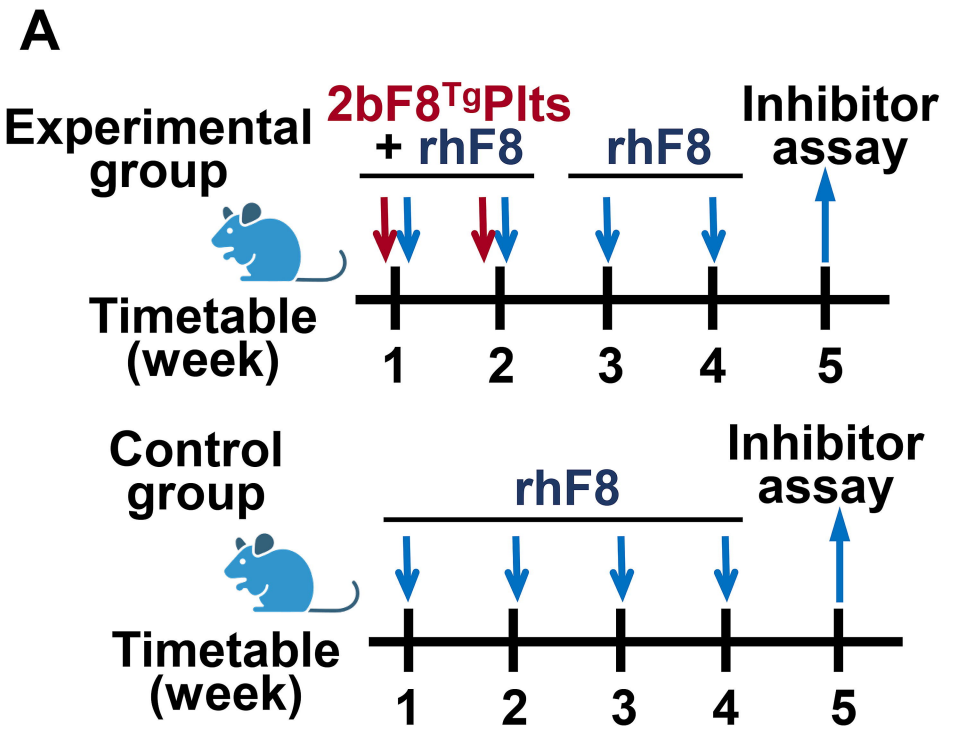
Figure 5. Desialylated platelet infusion expands T regulatory (Treg) cells and suppresses T cell proliferation. For Treg cell analysis, blood samples were collected via retro-orbital bleeds with 3.8% sodium citrate as an anticoagulant. Leukocytes were stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry. For the T cell proliferation assay, splenocytes were isolated from animals one week after the last recombinant FVIII (rhF8) immunization. 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 for the daughter cells from violet-labeled CD4 T cells. (**A**) Representative flow dot plots of Treg cells from a mouse receiving 2bF8^{Tg}dPlts transfusion and rhF8 immunization. (**B**) The percentages of Treg cells in FVIII^{null}(B6/129) mice before and after 2bF8^{Tg}dPlts transfusion, as well as after rhF8 immunization, are shown. (**C**) Treg cells in mice after rhF8 immunization with or without pre-sensitization with 2bF8^{Tg}dPlts transfusion. (**D**) Representative histograms of daughter CD4 T cells from the Violet-labeled T cell proliferation assay are shown. (**E**) The stimulation index of CD4 T cell proliferation in each group cultured with rhF8 is shown. The stimulation index (SI) was calculated as follows: SI = (the percentage of proliferating daughter cells in rhF8 or rhF9-treated wells) / (the percentage of proliferating daughter cells in control wells with 0 μg/ml of rhF8). Recombinant human factor IX (rhF9) was used as an unrelated control antigen. The data were summarized from two trials. *$P < 0.05$; **$P < 0.01$; "ns" indicates no statistically significant difference between the two groups by one-way nonparametric Friedman test or Paired *t*-test for (**B**), unpaired Student *t*-test for (**C**), or two-way ANOVA for (**E**).

Figure 6. The modulatory effect of platelets containing FVIII on FVIII immune responses in F8^{KO}(B6) mice. Platelets were isolated from 2bF8 transgenic (2bF8^{Tg}) or wild-type (Wt) mice. 2bF8^{Tg}Plts or WtPlts were infused into FVIII^{null} mice in a B6 background [F8^{KO}(B6)] along with recombinant human FVIII (rhF8 50 U/kg) *via* intravenous administration weekly for 4 weeks. The impact of desialylated 2bF8^{Tg}Plts (2bF8^{Tg}dPlts) on FVIII immune responses was also evaluated in this colony of mice, in which 2bF8^{Tg}dPlts were infused weekly for 4 weeks, followed by rhF8 50 U/kg/week for 4 weeks. F8^{KO}(B6) mice immunized with rhF8 alone served as controls. One week after the last rhF8 immunization, plasma samples were collected, and FVIII inhibitor titers were measured using the Bethesda assay, and anti-FVIII total IgG levels were determined by ELISA. (**A**) Diagram

of the experimental design for co-infusion of platelets and rhF8. (**B**) The incidence of FVIII inhibitor development. (**C**) FVIII inhibitor titers. (**D**) Anti-FVIII total IgG titers. Data are summarized from 5 infusion trials. *$P < 0.05$; **$P < 0.01$; “ns” indicates no statistically significant difference between the two groups by the one-way ANOVA.

Figure 1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2bF8TgPlts + rhF8
rhF8
Inhibitor assay
Timetable (week)
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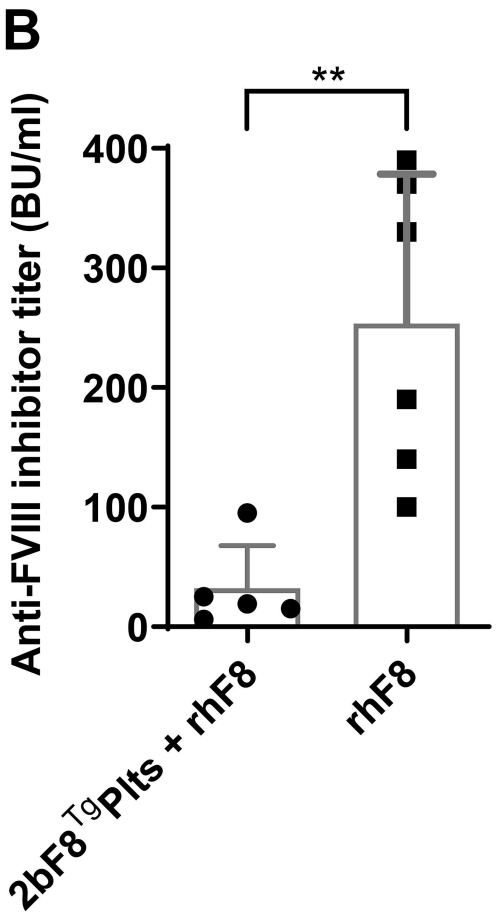
2
3
4
5
Control group
rhF8
Inhibitor assay
Timetable (week)
1
2
3
4
5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200
100
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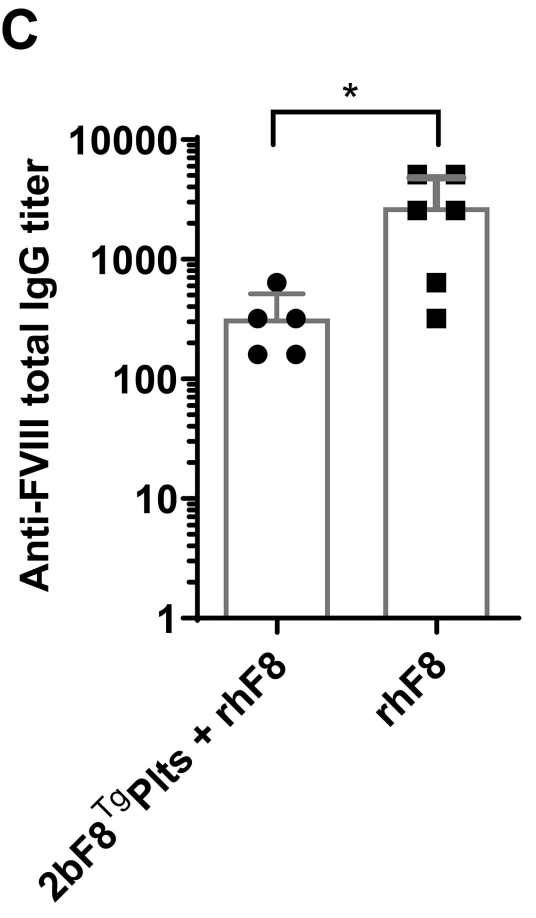
rhF8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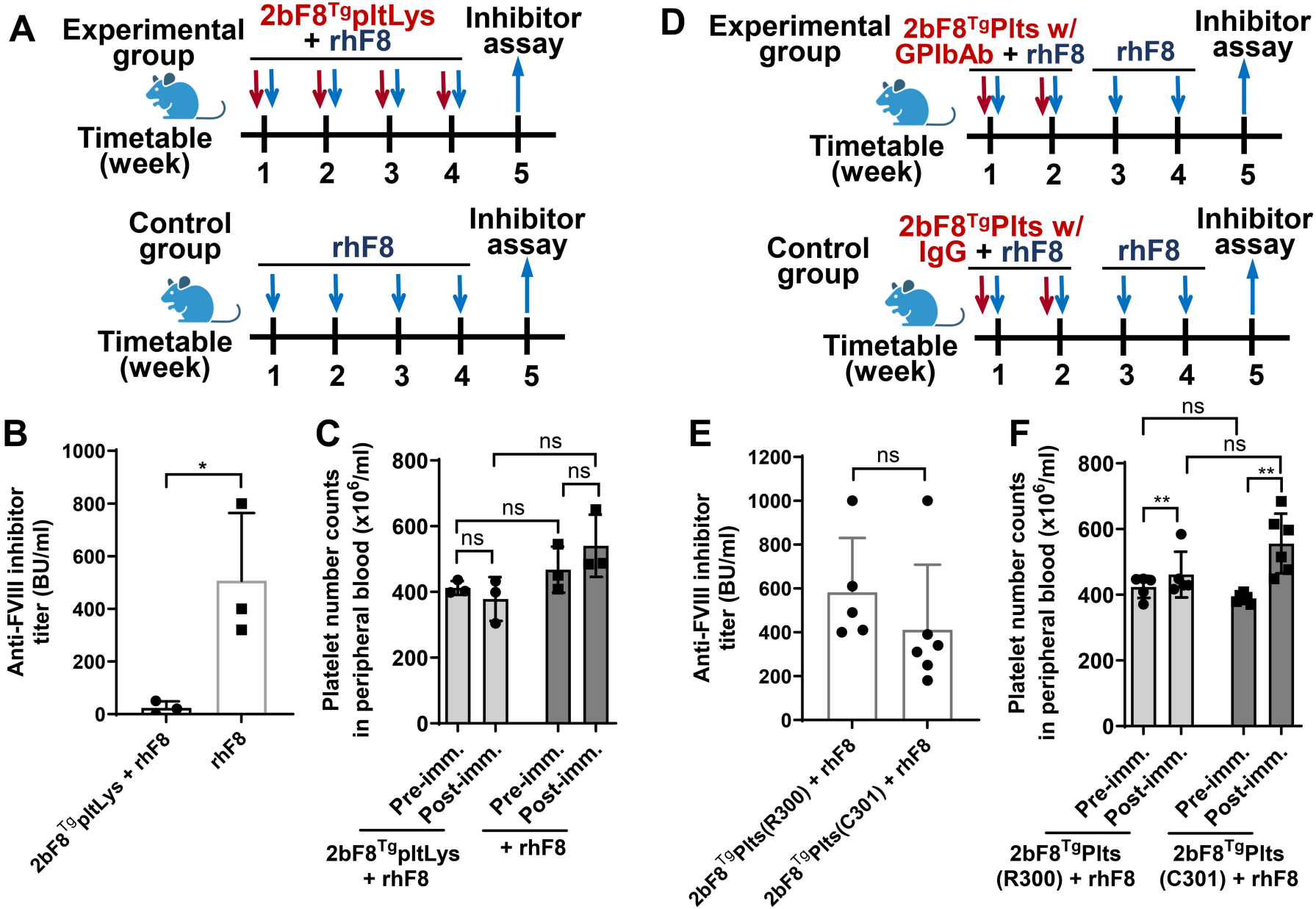
1000
100
10
1
2bF8TgPlts + rhF8
rhF8

Figure 2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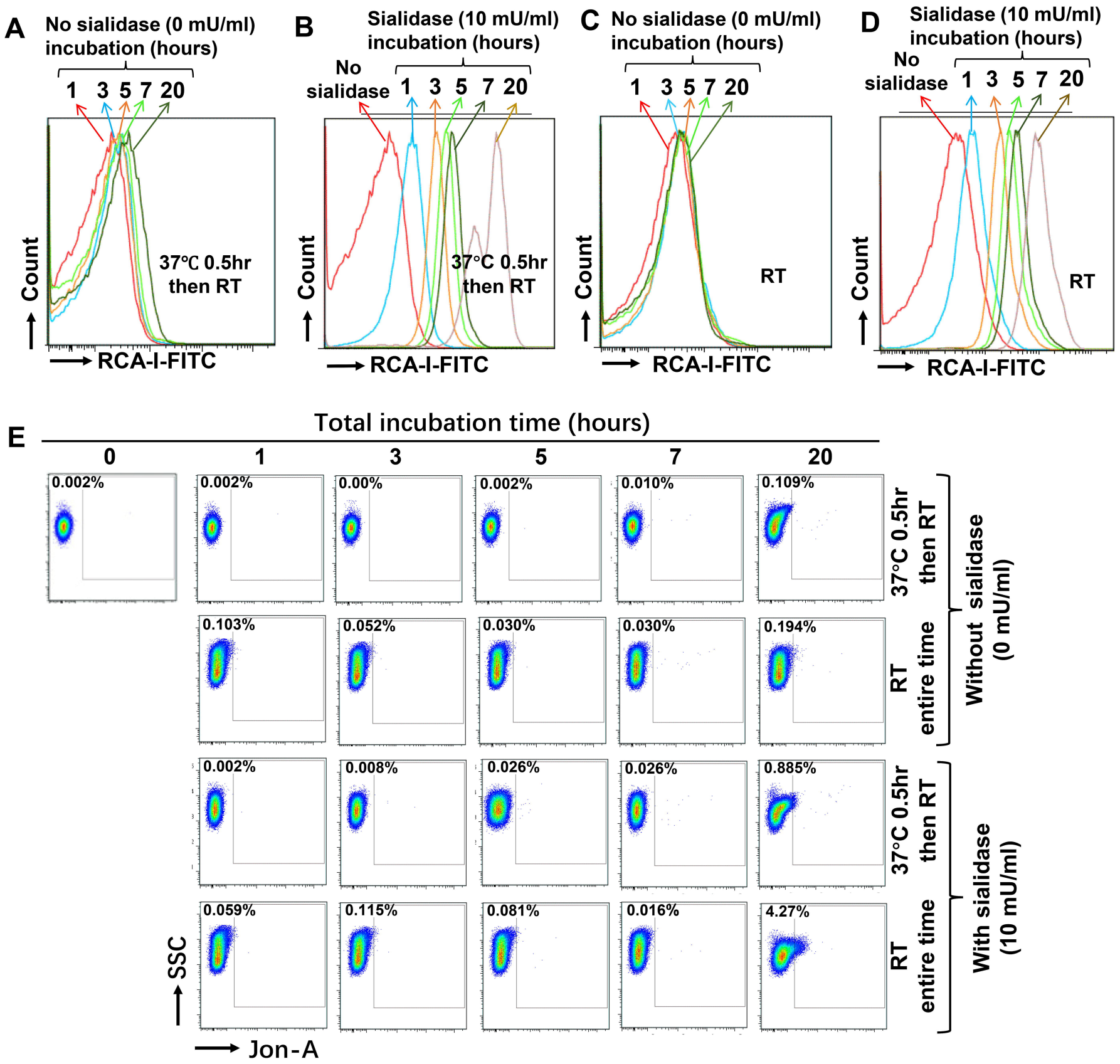
Inhibitor assay
Timetable (week)
1 2 3 4 5
Control group
rhF8
Inhibitor assay
Timetable (week)
1 2 3 4 5
D
Experimental group
2bF8TgPlts w/ GPIbAb + rhF8
rhF8
Inhibitor assay
Timetable (week)
1 2 3 4 5
Control group
2bF8TgPlts w/ IgG + rhF8
rhF8
Inhibitor assay
Timetable (week)
1 2 3 4 5
B
Anti-FVIII inhibitor titer (BU/ml)
1000
800
600
400
200
0
*
2bF8Tg pltLys + rhF8
rhF8
C
Platelet number counts in peripheral blood (x10^6/ml)
800
600
400
200
0
ns
ns
ns
ns
Pre-imm.
Post-imm.
Pre-imm.
Post-imm.
2bF8Tg pltLys + rhF8
+ rhF8
E
Anti-FVIII inhibitor titer (BU/ml)
1200
1000
800
600
400
200
0
ns
2bF8Tg Plts(R300) + rhF8
2bF8Tg Plts(C301) + rhF8
F
Platelet number counts in peripheral blood (x10^6/ml)
800
600
400
200
0
ns
ns
**
**
Pre-imm.
Post-imm.
Pre-imm.
Post-imm.
2bF8Tg Plts (R300) + rhF8
2bF8Tg Plts (C301) + rhF8

Figure 3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then RT
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incubation (hours)
No
sialidase
1 3 5 7 20
37°C 0.5hr
then RT
Count
RCA-I-FITC
C
No sialidase (0 mU/ml)
incubation (hours)
1 3 5 7 20
RT
Count
RCA-I-FITC
D
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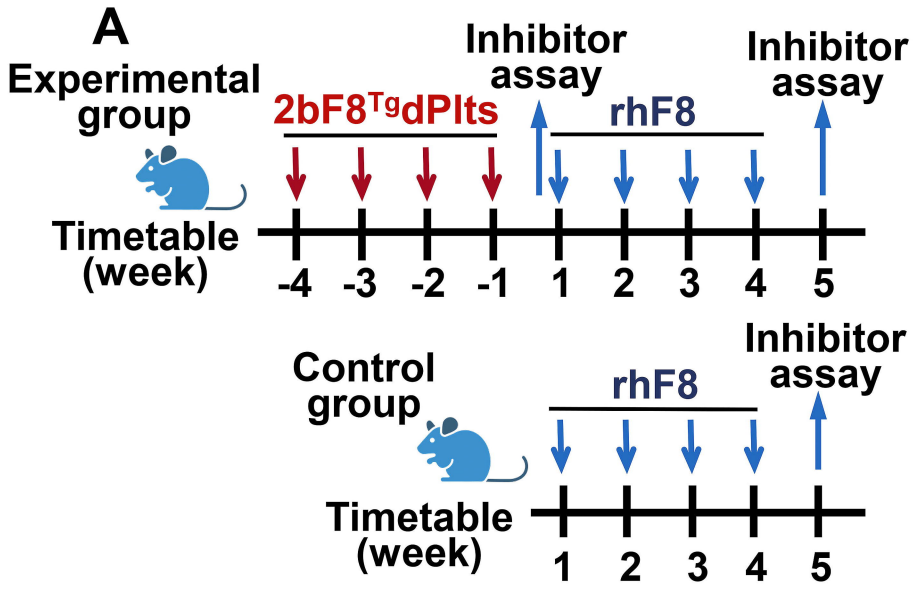
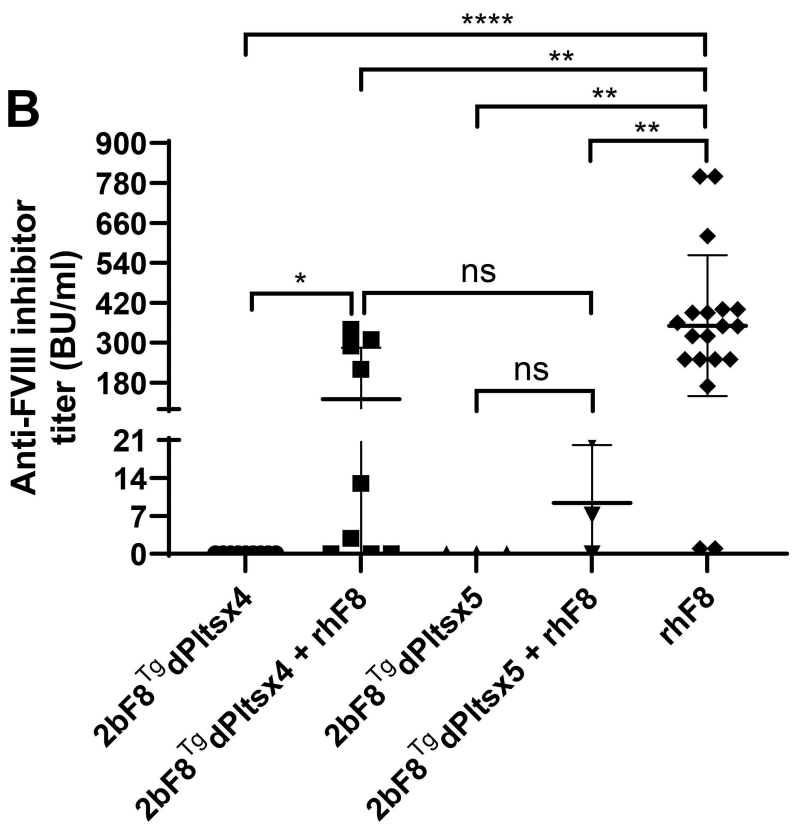
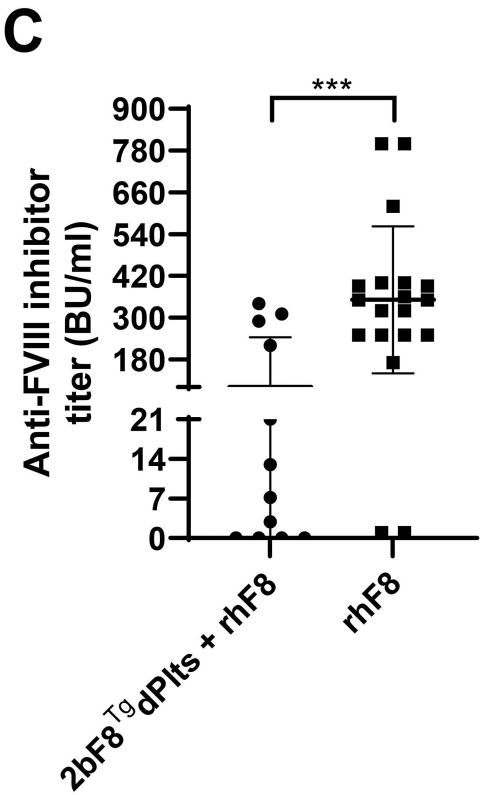
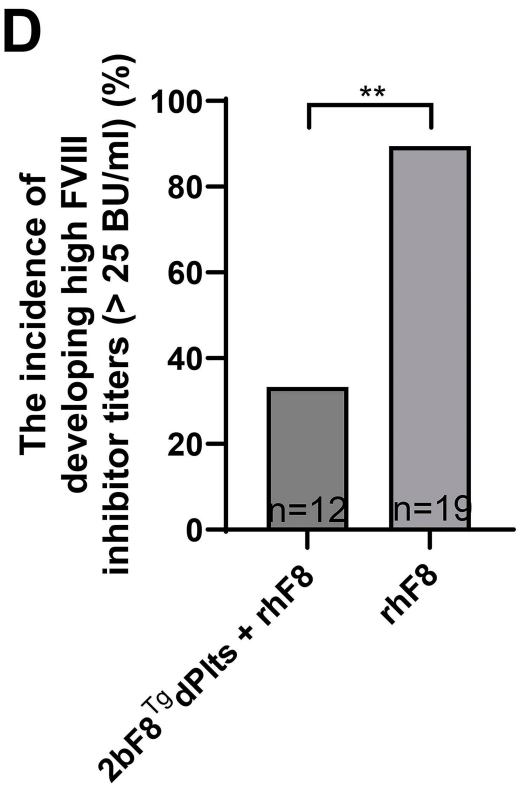
incubation (hours)
No
sialidase
1 3 5 7 20
RT
Count
RCA-I-FITC
E
Total incubation time (hours)
0
1
3
5
7
20
0.002%
0.002%
0.00%
0.002%
0.010%
0.109%
37°C 0.5hr
then RT
0.103%
0.052%
0.030%
0.030%
0.194%
RT
entire time
Without sialidase
(0 mU/ml)
0.002%
0.008%
0.026%
0.026%
0.885%
37°C 0.5hr
then RT
0.059%
0.115%
0.081%
0.016%
4.27%
RT
entire time
With sialidase
(10 mU/ml)
SSC
Jon-A

Figure 4


A
Experimental group
2bF8TgdPlts
Inhibitor assay
rhF8
Inhibitor assay
Timetable (week)
-4 -3 -2 -1 1 2 3 4 5
Control group
rhF8
Inhibitor assay
Timetable (week)
1 2 3 4 5


B
Anti-FVIII inhibitor titer (BU/ml)
900
780
660
540
420
300
180
21
14
7
0
****
**
**
**
*
ns
ns
2bF8TgdPltsx4
2bF8TgdPltsx4 + rhF8
2bF8TgdPltsx5
2bF8TgdPltsx5 + rhF8
rhF8


C
Anti-FVIII inhibitor titer (BU/ml)
900
780
660
540
420
300
180
21
14
7
0
***
2bF8TgdPlts + rhF8
rhF8


D
The incidence of developing high FVIII inhibitor titers (> 25 BU/ml) (%)
100
80
60
40
20
0
**
n=12
n=19
2bF8TgdPlts + rhF8
rhF8

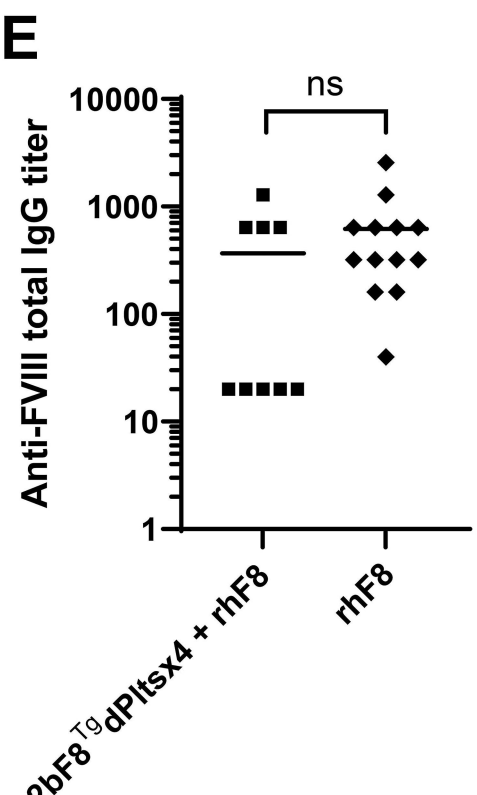

E
Anti-FVIII total IgG titer
10000
1000
100
10
1
ns
2bF8TgdPltsx4 + rhF8
rhF8

Figure 5

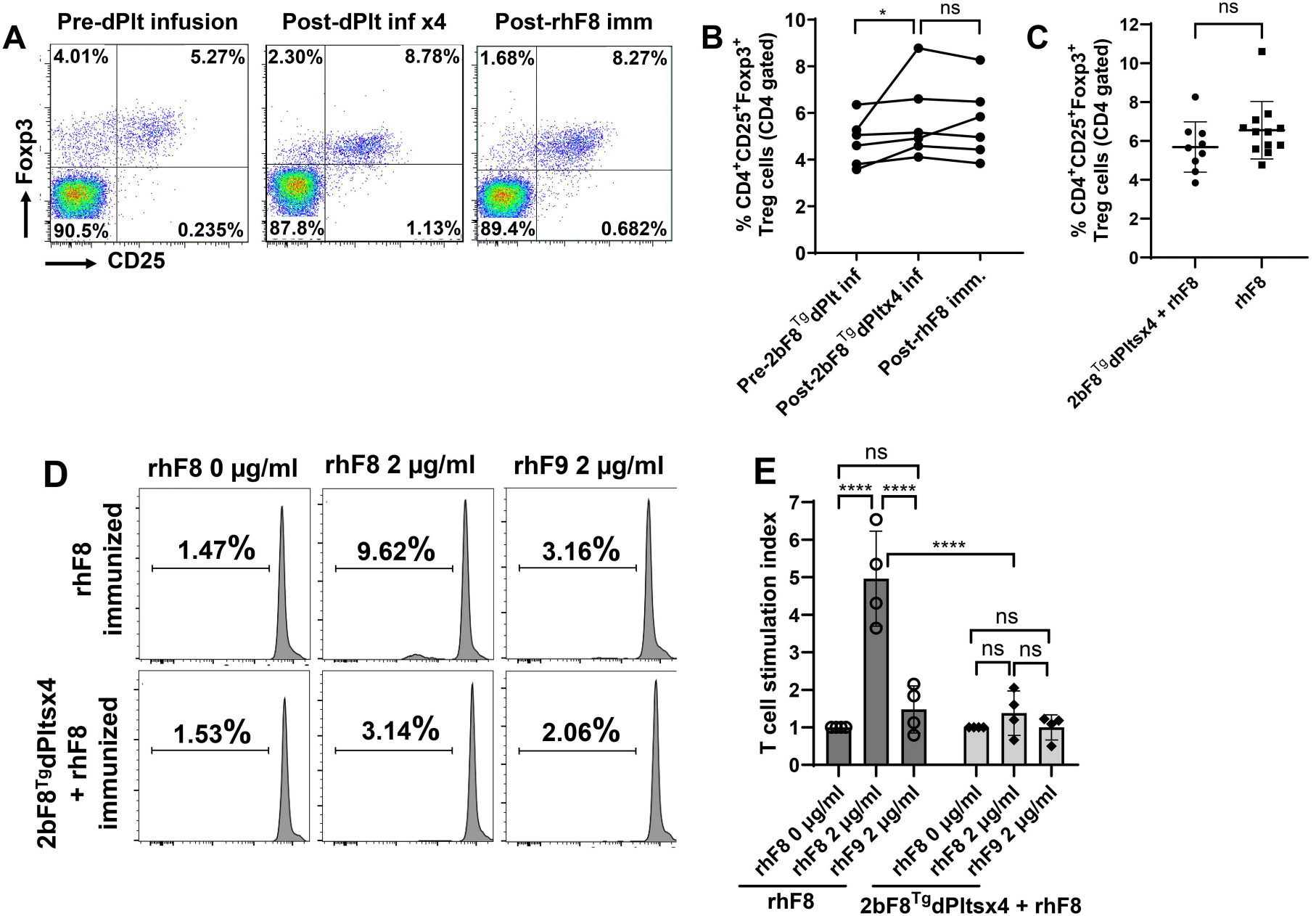
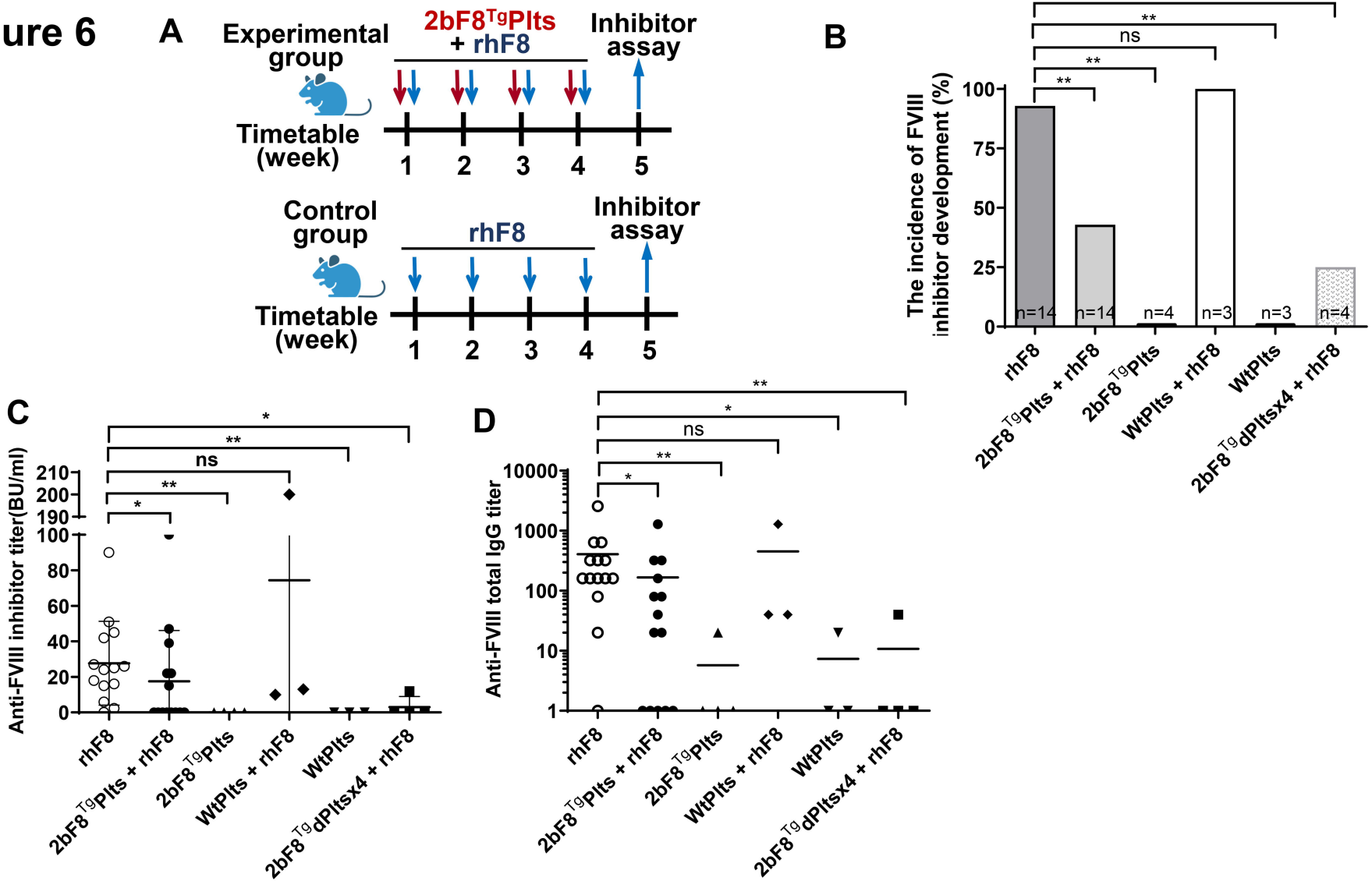

A
Pre-dPlt infusion
Post-dPlt inf x4
Post-rhF8 imm
4.01%
5.27%
90.5%
0.235%
2.30%
8.78%
87.8%
1.13%
1.68%
8.27%
89.4%
0.682%
Foxp3
CD25
B
% CD4+CD25+Foxp3+ Treg cells (CD4 gated)
*
ns
Pre-2bF8 Tg dPlt inf
Post-2bF8 Tg dPltx4 inf
Post-rhF8 imm.
C
% CD4+CD25+Foxp3+ Treg cells (CD4 gated)
ns
2bF8 Tg dPltsx4 + rhF8
rhF8
D
rhF8 0 µg/ml
rhF8 2 µg/ml
rhF9 2 µg/ml
rhF8 immunized
2bF8Tg dPltsx4 + rhF8 immunized
1.47%
9.62%
3.16%
1.53%
3.14%
2.06%
E
T cell stimulation index
ns
****
****
****
ns
ns
ns
rhF8 0 µg/ml
rhF8 2 µg/ml
rhF9 2 µg/ml
rhF8 0 µg/ml
rhF8 2 µg/ml
rhF9 2 µg/ml
rhF8
2bF8Tg dPltsx4 + rhF8

Figure 6


A
Experimental group
2bF8TgPlts + rhF8
Inhibitor assay
Timetable (week)
1 2 3 4 5
Control group
rhF8
Inhibitor assay
Timetable (week)
1 2 3 4 5
B
The incidence of FVIII inhibitor development (%)
100
75
50
25
0
n=14
n=14
n=4
n=3
n=3
n=4
ns
*
**
rhF8
2bF8TgPlts + rhF8
2bF8TgPlts
WtPlts + rhF8
WtPlts
2bF8TgdPltsx4 + rhF8
C
Anti-FVIII inhibitor titer(BU/ml)
210
200
190
100
80
60
40
20
0
ns
*
**
rhF8
2bF8TgPlts + rhF8
2bF8TgPlts
WtPlts + rhF8
WtPlts
2bF8TgdPltsx4 + rhF8
D
Anti-FVIII total IgG titer
10000
1000
100
10
1
ns
*
**
rhF8
2bF8TgPlts + rhF8
2bF8TgPlts
WtPlts + rhF8
WtPlts
2bF8TgdPltsx4 + rhF8

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.

References

1. Chen Y, Schroeder JA, Chen J, et al. The immunogenicity of platelet-derived FVIII in hemophilia A mice with or without preexisting anti-FVIII immunity. Blood 2016;127(10):1346-1354.
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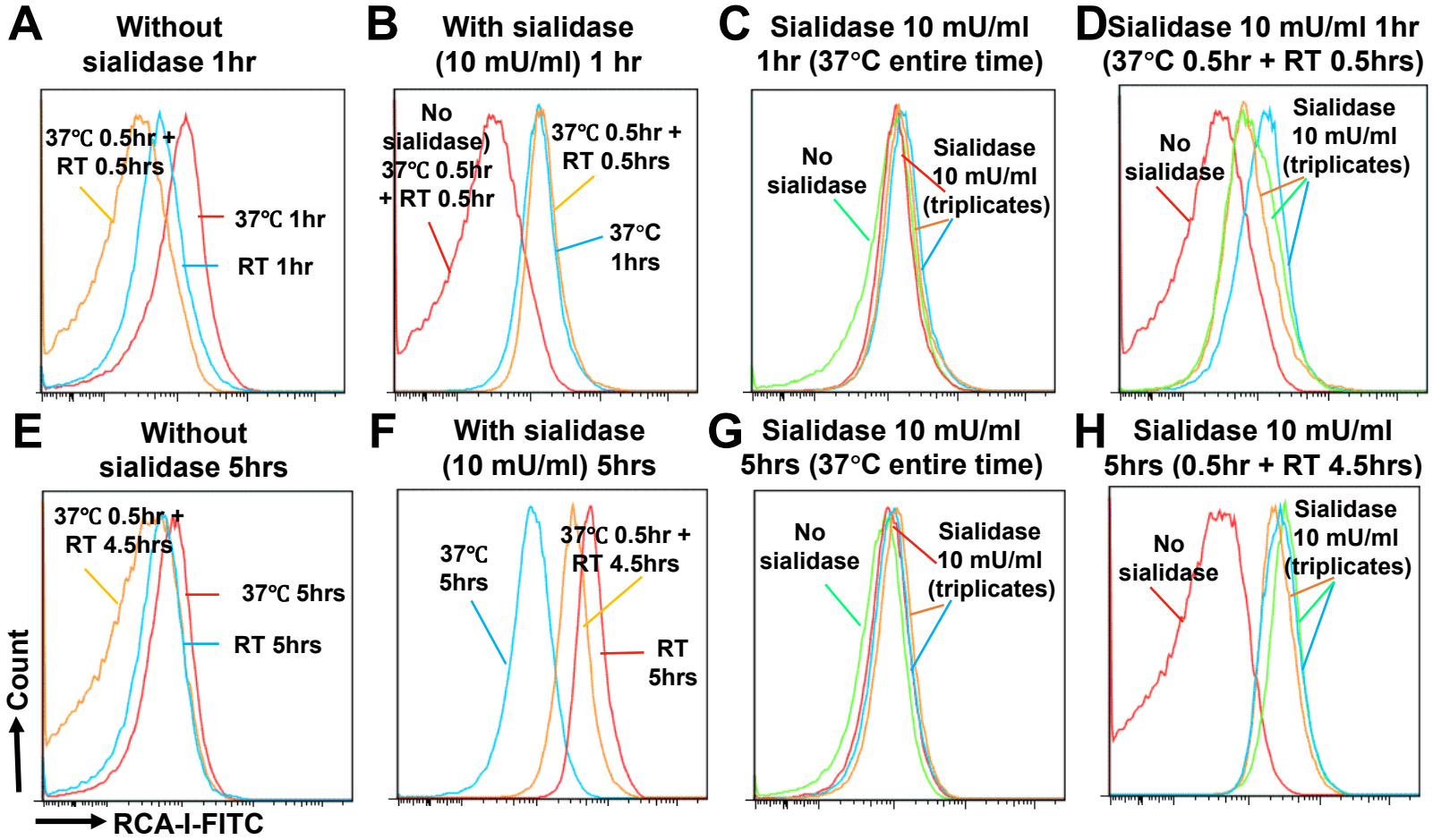
Supplemental Table 1. The abbreviations used in this study

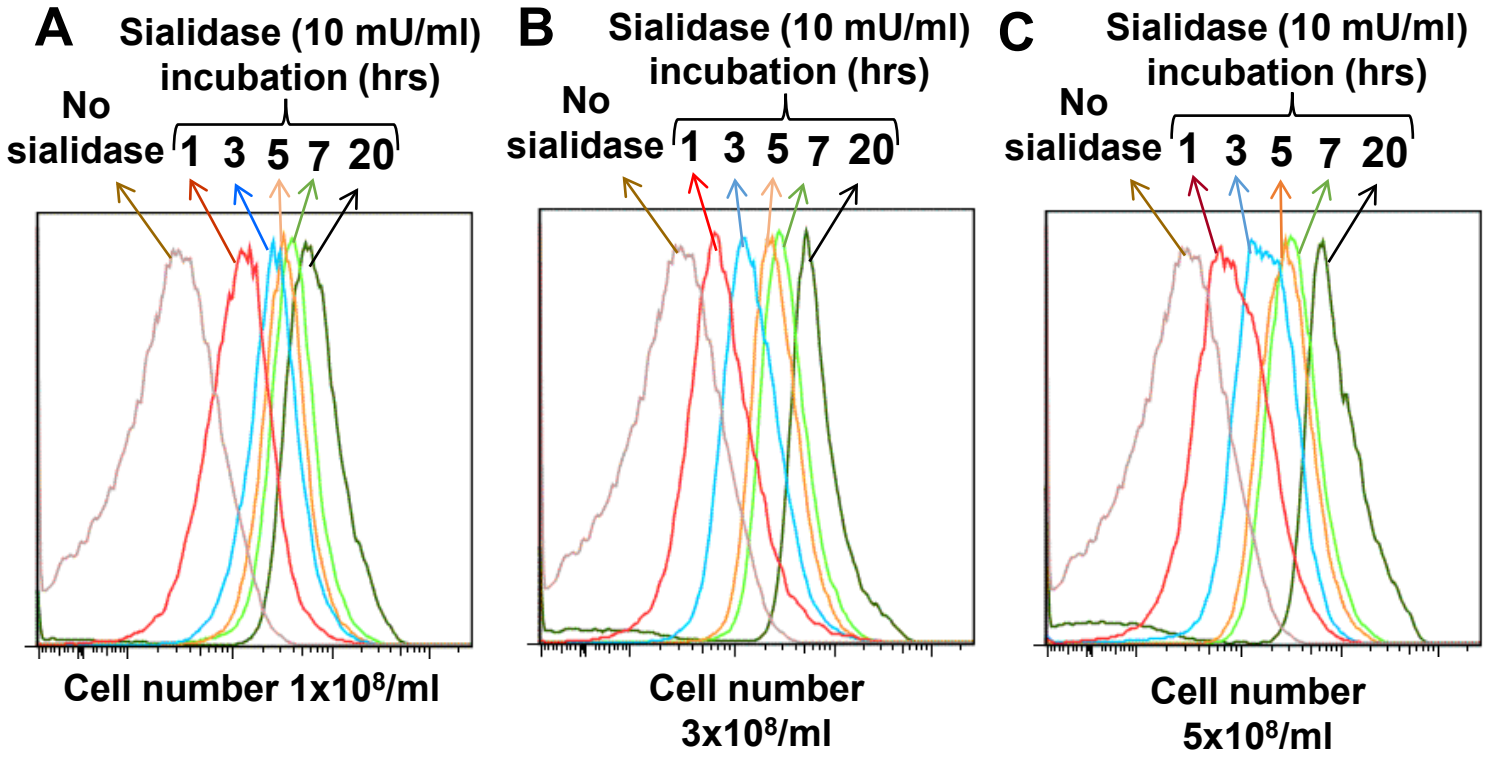
Abbreviation	Definition
HA	hemophilia A
FVIII	factor VIII
FIX	factor IX
FX	factor X
VWF	von Willebrand factor
rhF8	recombinant human FVIII
rhF9	recombinant human FIX
2bF8	human B-domain-deleted FVIII expression under the control of the platelet-specific αIIb promoter
$2bF8^{Tg}$	2bF8 transgenic mice
$2bF8^{Tg}$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



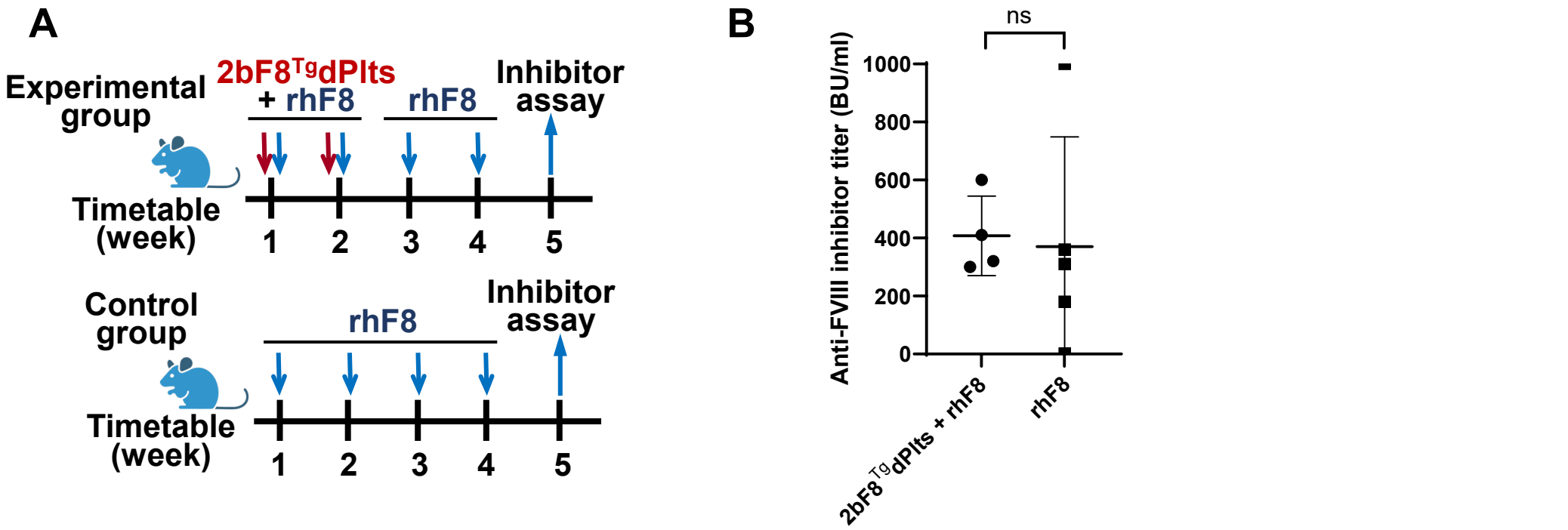
Supplemental Figure 1. Optimization of desialylating conditions to maximize platelet desialylation. Blood samples were collected from 2bF8^{Tg} mice, and platelets were isolated. Platelets (5 x 10^8/ml) were treated with 10 mU/ml of neuraminidase in modified Tyrode buffer, then incubated at various temperatures [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



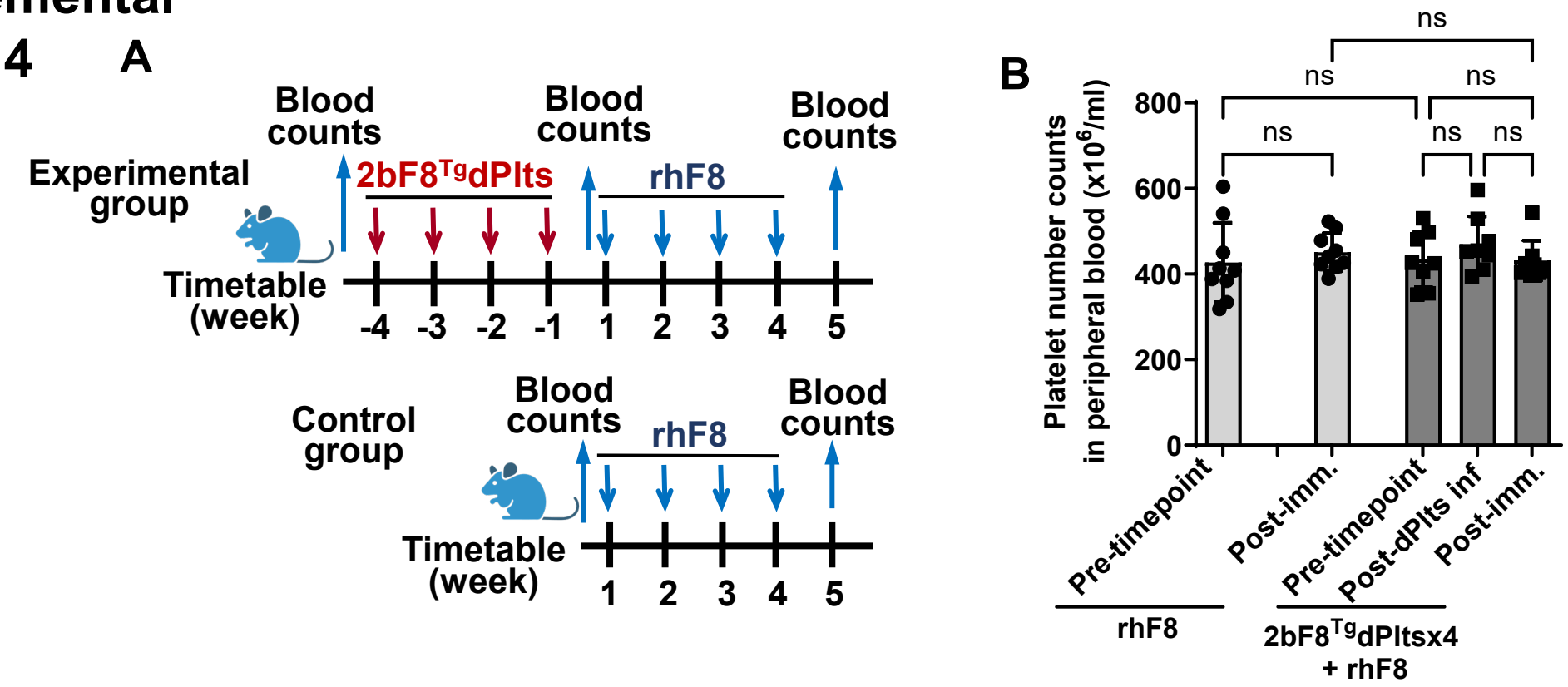
Supplemental Figure 2. Assessing the desialylation efficacy on various platelet number under our optimized desialylating conditions. Blood samples were collected from 2bF8^{Tg} mice, and platelets were isolated. Various platelet numbers (1-5 x 10^8/ml) were treated with 10 mU/ml of neuraminidase in modified 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



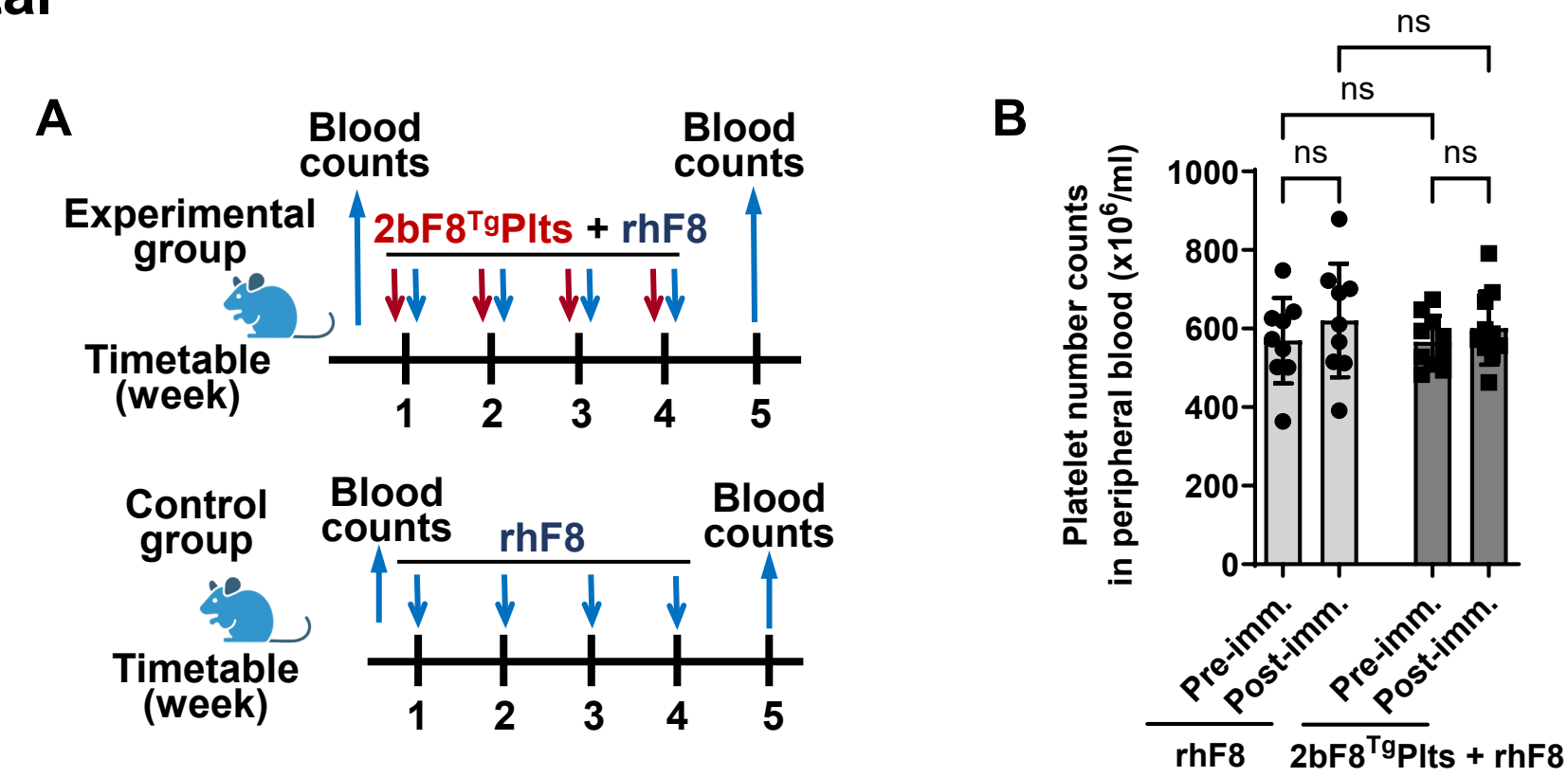
Supplemental Figure 3. The effect of co-infusion of desialylated platelets containing FVIII on FVIII immune responses in FVIII^{null}(129/B6) mice. Platelets were isolated from 2bF8 transgenic (2bF8^{Tg}) mice. 2bF8^{Tg}Plts were desialylated and infused into FVIII^{null} mice in a B6/129 mixed background [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



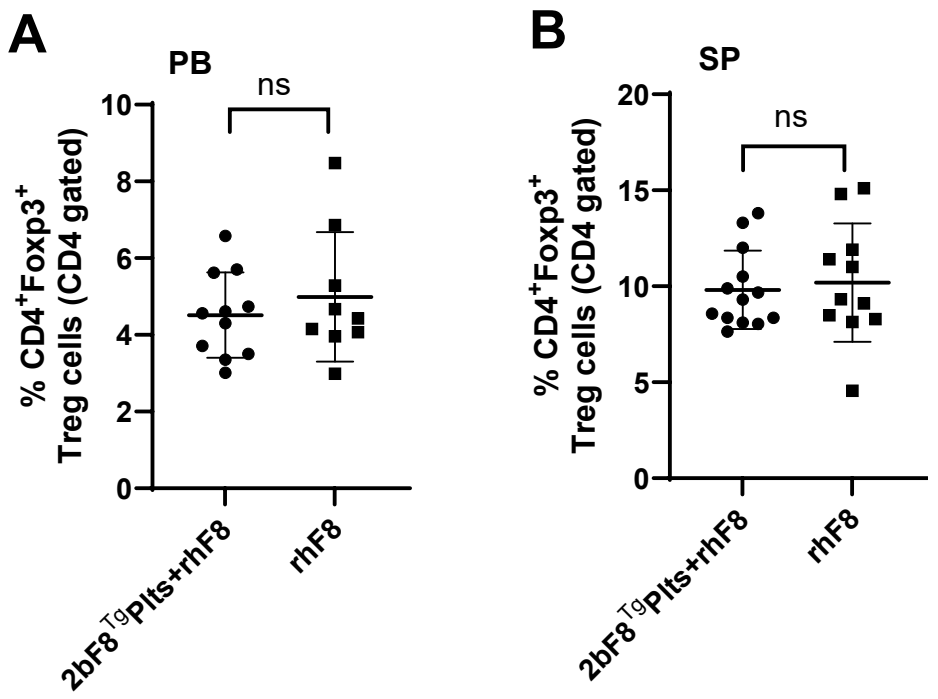
Supplemental Figure 4. The influence of transfusion of desialylated 2bF8^{Tg} platelets (2bF8^{Tg}dPlts) in FVIII^{null}(B6/129) mice. Blood samples were collected from FVIII^{null}(B6/129) mice before and after receiving 2bF8^{Tg}dPlts transfusions and after receiving recombinant human FVIII (rhF8) 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.