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

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Abstract

Development of inhibitory antibodies (inhibitors) against factor VIII (FVIII) is a significant complication of protein replacement therapy in hemophilia A (HA). Platelets (Plt), 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. The object was to investigate whether FVIII-engineered Plt and related Plt-based products modulate FVIII immune responses in HA mice. FVIII-containing Plt were isolated from 2bF8 transgenic mice. FVIII-deficient mice were infused with intact FVIII-containing Plt, desialylated FVIII-containing Plt (dPlt), or acidified Plt lysates in combination with or before recombinant human FVIII (rhF8) exposure. Anti-FVIII antibody titers were determined by the Bethesda assay and enzyme-linked immunosorbent assay, and T-cell responses were analyzed by flow cytometry and proliferation assays. Co-infusion of FVIII Plt with rhF8 significantly reduced inhibitor titers compared with rhF8 alone. Acidified FVIII Plt lysates were potent, decreasing inhibitor titers by >20-fold when co-infused with rhF8. In contrast, co-infusion of dPlt with rhF8 did not suppress immune responses. However, repeated pre-sensitization with dPlt 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 Plt providing partial protection, lysates strongly suppressing immune responses, and dPlt inducing immune tolerance. FVIII-engineered Plt and Plt-derived products are potent immune modulators. These strategies offer novel and translatable approaches to both restore hemostasis and prevent or eradicate inhibitors in HA.

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 emici-

zumab 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 (Plt) 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 Plt 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] Plt-derived TGF-β1 is a potent inducer of regulatory T cells (Treg), crucial for immune regulation.[17] Aged Plt 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 Plt can uniquely deliver therapeutic proteins and regulate immune responses. Leveraging these unique properties, our group developed Plt-targeted FVIII gene therapy using a Plt-specific promoter (αIIb) to drive FVIII (2bF8) expression, thereby storing FVIII with VWF in Plt α-granules and releasing it at the sites of injury.[20] Our studies demonstrate that Plt-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 Plt could actively modulate FVIII immune responses and prevent inhibitor formation. We focused on three approaches in FVIII-deficient mice: (i) co-infusion of FVIII-engineered Plt with recombinant human FVIII (rhF8) to test their effect during initial FVIII exposure, (ii) administration of acidified FVIII Plt lysates to evaluate the role of Plt-derived regulatory factors, and (iii) infusion of desialylated FVIII-engineered Plt (dPlt) to direct clearance through tolerogenic pathways. Our findings demonstrate that FVIII-engineered Plt 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 *Online Supplementary Appendix*. Abbreviations used are listed in *Online Supplementary Table S1*.

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 Plt-specific αIIb promoter (2bF8)[25] and used as donors for Plt 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 Plt infusion and rhF8 immunization. Wild-type (WT) C57BL/6 mice were used to isolate WT Plt (WtPlt) as controls.

Platelet isolation, desialylation, and infusion

Blood sample collection and Plt isolation were performed as reported.[28] Isolated Plt were transfused into FVIII^{null} mice, and animals were immunized with rhF8. dPlt were prepared using neuraminidase, washed, and resuspended in Tyrode buffer at a concentration of 2x10^9 Plt/mL. To determine the percentage of dPlt, neuraminidase-treated platelets were stained with RCA-I and analyzed by fluoreascense-activated cell sorting (FACS). JON/A antibody was used to stain activated Plt integrin α2bβ3 as reported.[29] dPlt 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.

Plt lysates from 2bF8^{Tg} mice (2bF8^{Tg}pltLys) were prepared by freezing and thawing Plt, as previously reported.[30] The amount of TGF-β1 in Plt lysates (pltTGF-β1) was measured using enzyme-linked immunosorbant assay (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 Plt pretreated with anti-GPIb antibody experiments, Plt isolated from 2bF8^{Tg} mice were pre-incubated with anti-GPIb antibody (R300, Emfret) at a dose of 2μg/10^8 Plt. IgG isotype (C301) was used as a control in parallel. Antibody-coated 2bF8^{Tg}Plt were infused along with rhF8 into FVIII^{null}(B6/129) mice weekly for 2 weeks, followed by 2 additional weeks of rhF8 infusions.

FVIII immune response studies

HA mice with or without the infusion of 2bF8^{Tg}Plt, dPlt, 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 1 week after the last immunization. Plt 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}) Plt 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} Plt with rhF8 affects FVIII immune responses, we transfused 2bF8^{Tg} Plt 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 2 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} Plt plus rhF8 co-infused (2bF8^{Tg}Plt + 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}Plt and rhF8 co-infusion group was also significantly lower than in the rhF8 group (320±196 *vs*. 2,720±2,081) (Figure 1C). These results demonstrated that co-infusion of Plt 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 Plt.[30] Acidified TGF-β1 from Plt lysates (pltLys) effectively induces Treg cells (iTreg), which suppress FVIII immune responses better than purified TGF-β1-induced Treg 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 Plt. 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 *vs*. 506.7±257.2 BU/mL). Plt 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* Plt 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 Plt coupled with IbAb *in vitro*, then transfuse them to mimic *in vivo* Plt deletion and modulate FVIII immune responses. We tested whether co-infusing 2bF8^{Tg}Plt pre-incubated with R300, along with rhF8 infusions, affects FVIII immune responses. We incubated isolated 2bF8^{Tg}Plt with R300 (2μg/10^8 Plt) to simulate an *in vivo* dose of 1 mg/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}Plt (2bF8^{Tg}Plts[R300]) *versus* IgG isotype-treated 2bF8^{Tg}Plt(C301). Interestingly, Plt counts were significantly elevated after

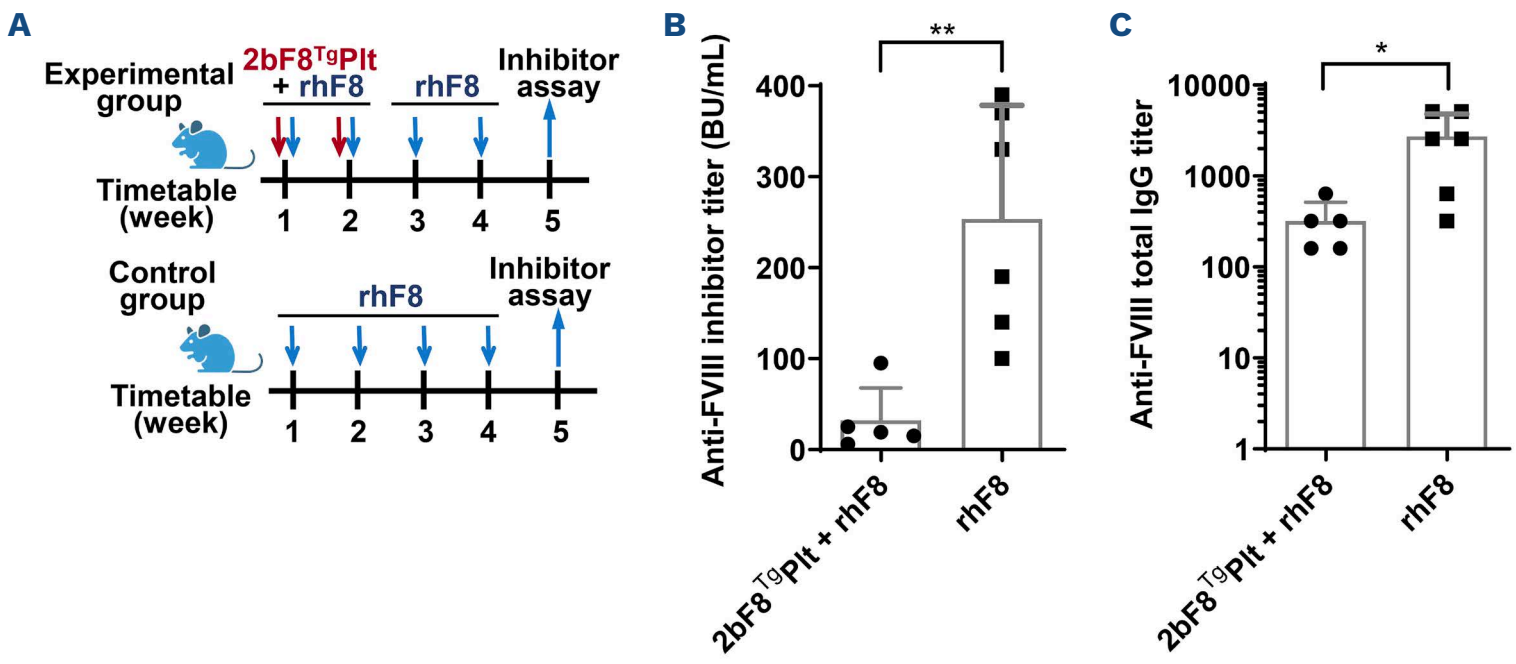


Figure 1. The modulatory effect of platelets containing FVIII on FVIII immune responses in FVIII^{null}(B6/129) mice. Platelets (Plt) were isolated from 2bF8 transgenic (2bF8^{Tg}) mice. 2bF8^{Tg}Plt were infused into FVIII^{null} mice in a B6/S129 mixed background (FVIII^{null}(B6/129)) along with recombinant human FVIII (rhF8) via intravenous administration weekly for 2 weeks, followed by an additional 2 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 enzyme-linked immunosorbent assay. (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}Plt infusion studies. *P<0.05; **P<0.01 by the unpaired Student t test.

infusions of R300- or C301-opsonized Plt (Figure 2F). These results demonstrate that acidified 2bF8^{Tg}pltLys can attenuate FVIII inhibitor development, consistent with activation of Plt-derived TGF-β1, whereas infusion of anti-GPIb-coated 2bF8^{Tg}Plt did not affect inhibitor titers but was associated with increased peripheral Plt counts.

Infusion of desialylated 2bF8^{Tg}Plt 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 Plt undergo apoptosis while their glycan proteins are desialylated,[19] creating an immunomodulatory environment *in vivo*.[18] Here, we evaluate the impact of dPlt that contain FVIII (2bF8^{Tg}dPlt) on FVIII immune responses in HA mice. First, we optimized our Plt desialylation protocol to ensure that greater than 95% of Plt were desialylated while remaining inactivated. We tested various concentrations of sialidase (neuraminidase) and Plt numbers under different incubation conditions. Interestingly, we found that incubating Plt at 37°C for more than 1 hour induced some degree of desialylation, and neuraminidase became ineffective (*Online Supplementary Figure S1A, C, E, G*). Incubating at

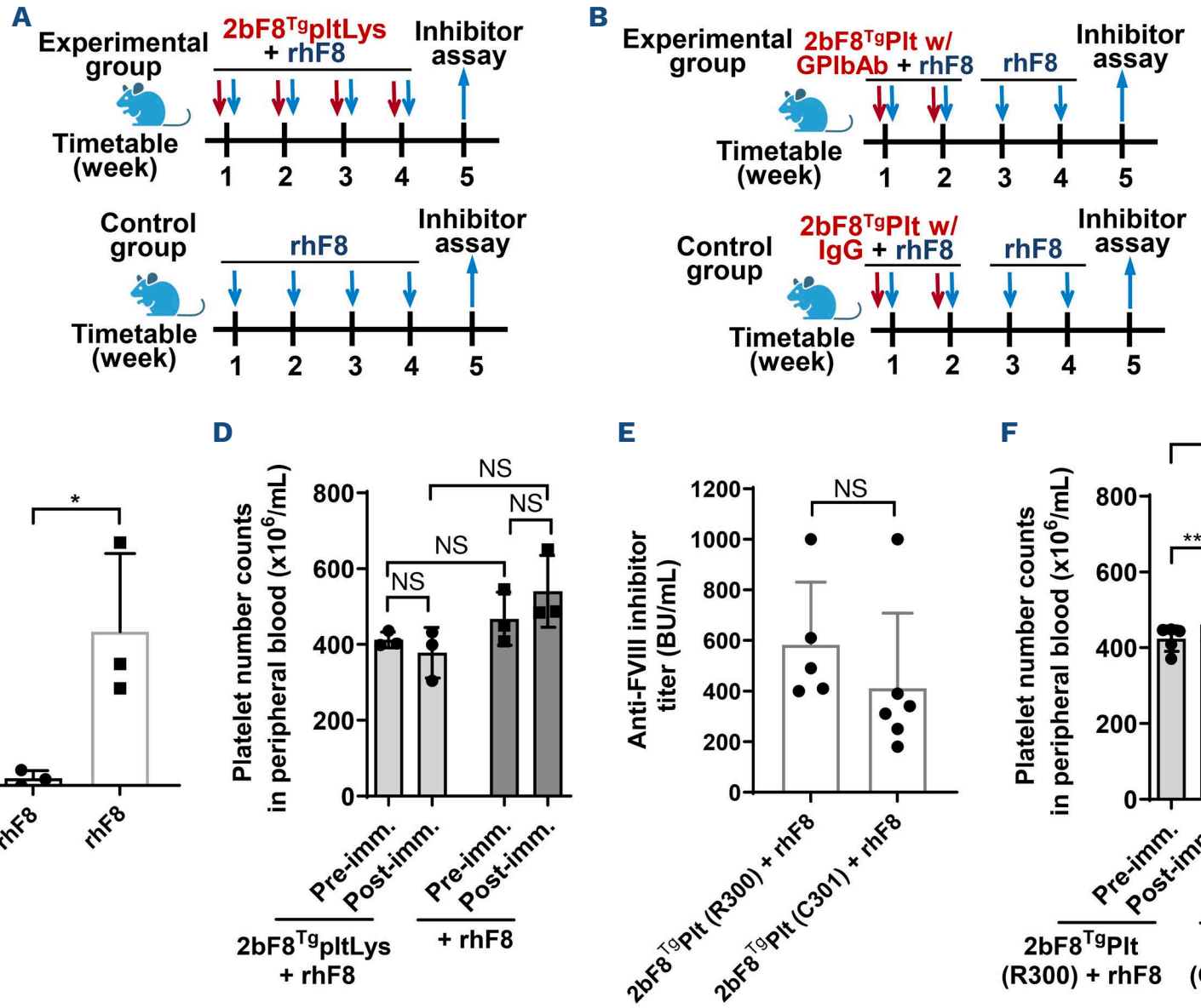
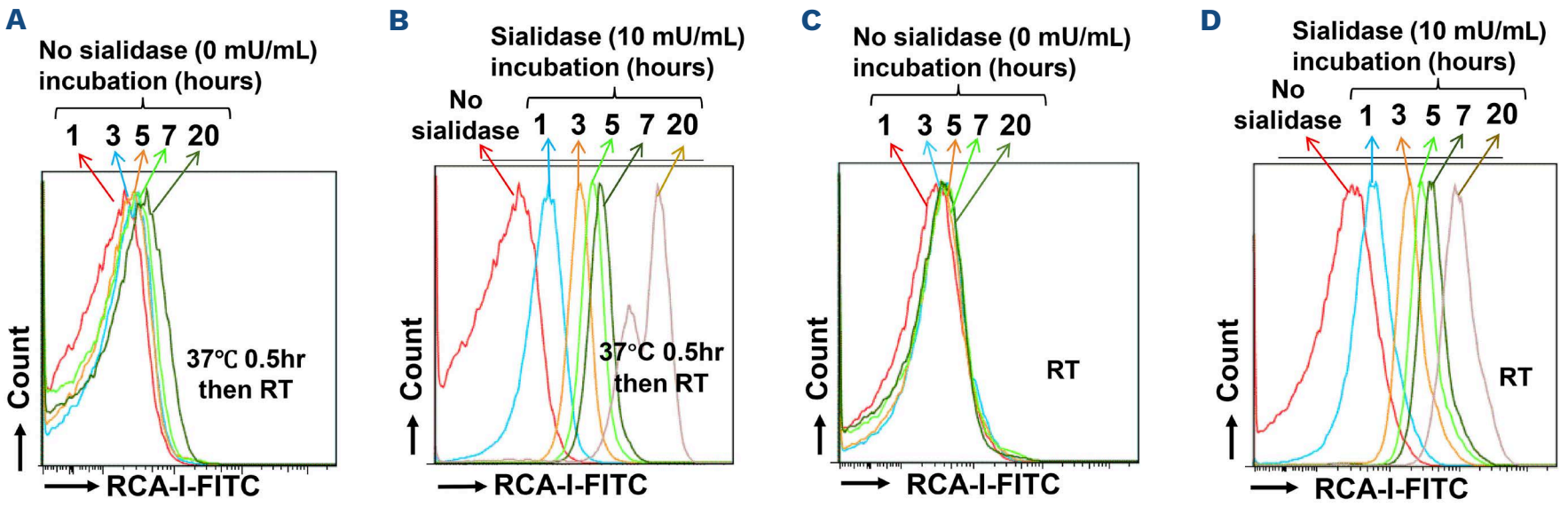


Figure 2. The modulatory effect of lysates from 2bF8^{Tg} platelets or anti-GPIb antibody-coated 2bF8^{Tg} platelets on FVIII immune responses in FVIII^{null}(129/B6) mice. Blood samples were collected from 2bF8 transgenic (2bF8^{Tg}) mice, and platelets (Plt) were isolated. Plt lysates (2bF8^{Tg}pltLys) 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 pltLys 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 Plt infusion, 2bF8^{Tg} Plt were pre-incubated with either anti-GPIb antibody or isotype control (C301) at a concentration of 2 μg per 10^8 Plt for 40 minutes. Antibody-incubated Plt 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) Plt 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}Plt infusion study. (E) FVIII inhibitor titers in the anti-GPIb antibody-coated 2bF8^{Tg}-Plt infusion study. (F) Plt 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}Plt. (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}Plt infusion studies. *P< 0.05; "NS" indicates no statistically significant difference between the 2 groups by the unpaired Student t test (B and E) or two-way ANOVA (C and F).

room temperature (RT) also caused desialylation, although to a lesser extent than at 37°C, and adding neuraminidase could further desialylate Plt (*Online Supplementary Figure S1A, B, E, F*). Incubating at 37°C for 0.5 hour, followed by RT incubation for an additional 4.5 hours, could maximize sialidase activity (*Online Supplementary Figure S1D, H;* Figure 3A-D) while still protecting Plt from activation (Figure 3E). With 10 mU/mL of neuraminidase following our optimized



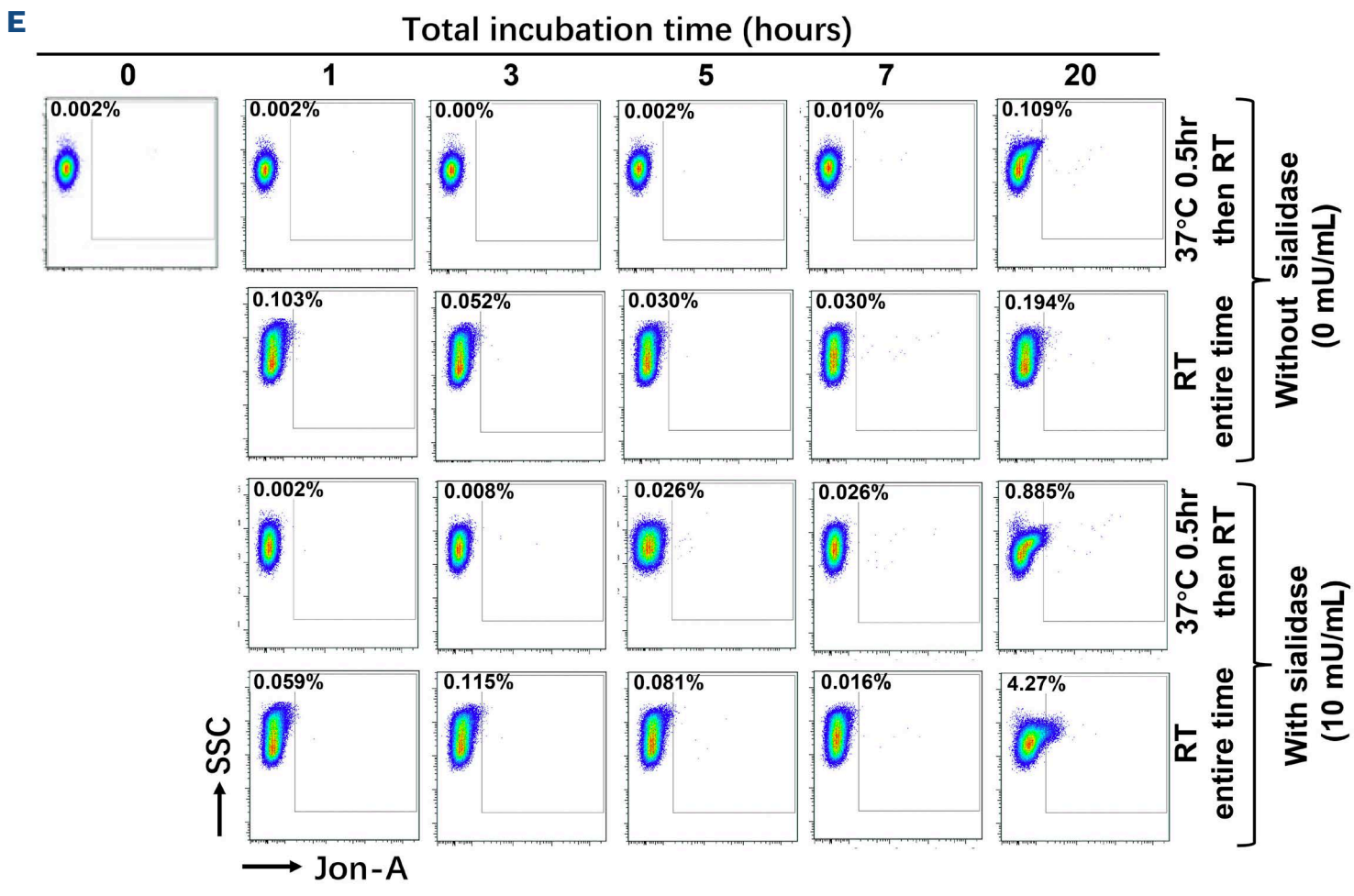


Figure 3. Optimization of desialylating conditions to maximize platelet desialylation while minimizing platelet activation. Blood samples were collected from 2bF8 transgenic (2BF8^{Tg}) mice, and platelets (Plt) were isolated. Plt (5x10^8/mL) were treated with 10 mU/mL of α2-3,6,8,9-neuraminidase in modified Tyrode buffer, then incubated at various temperatures (room temperature [RT], 37°C, or both) for different durations. After incubation, the Plt were washed and resuspended in Tyrode buffer. The desialylation levels in Plt were analyzed by flow cytometry after staining with Fluorescein Ricinus Communis agglutinin I (RCA-I). Plt activation levels were assessed by JON/A antibody staining and flow cytometry. (A) Baseline desialylation levels in 2bF8^{Tg}Plt without added neuraminidase, incubated at 37°C for 0.5 hour, followed by further incubation at room temperature. (B) Desialylation levels in 2bF8^{Tg}Plt with 10 mU/mL neuraminidase, incubated at 37°C for 0.5 hour, followed by further incubation at RT. The condition without adding sialidase, but incubating at 37°C for 0.5 hours, followed by incubation at RT for a total of 1 hour, served as the baseline control. (C) Baseline desialylation levels in 2bF8^{Tg}Plt without added neuraminidase, incubated at RT for varying durations of hours. (D) Desialylation levels in 2bF8^{Tg}Plt with 10 mU/mL neuraminidase, incubated at RT for varying durations of hours. The condition without adding sialidase but incubating at RT for 1 hour was used as a baseline control. (E) Activation levels in Plt after incubating with or without neuraminidase under varying incubation conditions. Plt were stained with JON-A antibody, which labels activated αIIbβ3, and analyzed by flow cytometry.

incubation condition, a cell number between 1-5x10^8 Plt/mL could be effectively desialylated (*Online Supplementary Figure S2*).

Using our optimal protocol, we evaluated how 2bF8^{Tg}dPlt infusion affects FVIII immune responses in HA mice. We first co-infused 2bF8^{Tg}dPlt together with rhF8 weekly into FVIII^{null}(B6/129) mice for 2 weeks, followed by an additional 2 weeks of rhF8 immunizations. We found no significant difference in FVIII inhibitor titers in mice that received 2bF8^{Tg}dPlt co-infusion *versus* the rhF8 groups (*Online Supplementary Figure S3*). We wondered whether it takes time to sensitize the immune system with 2bF8^{Tg}dPlt before exposing it to rhF8. We infused 2bF8^{Tg}dPlt alone, without rhF8, weekly for 4-5 weeks as an alternative Plt-based immune modulation strategy, then challenged with rhF8 weekly for 4 weeks (Figure 4A). We found that Plt counts

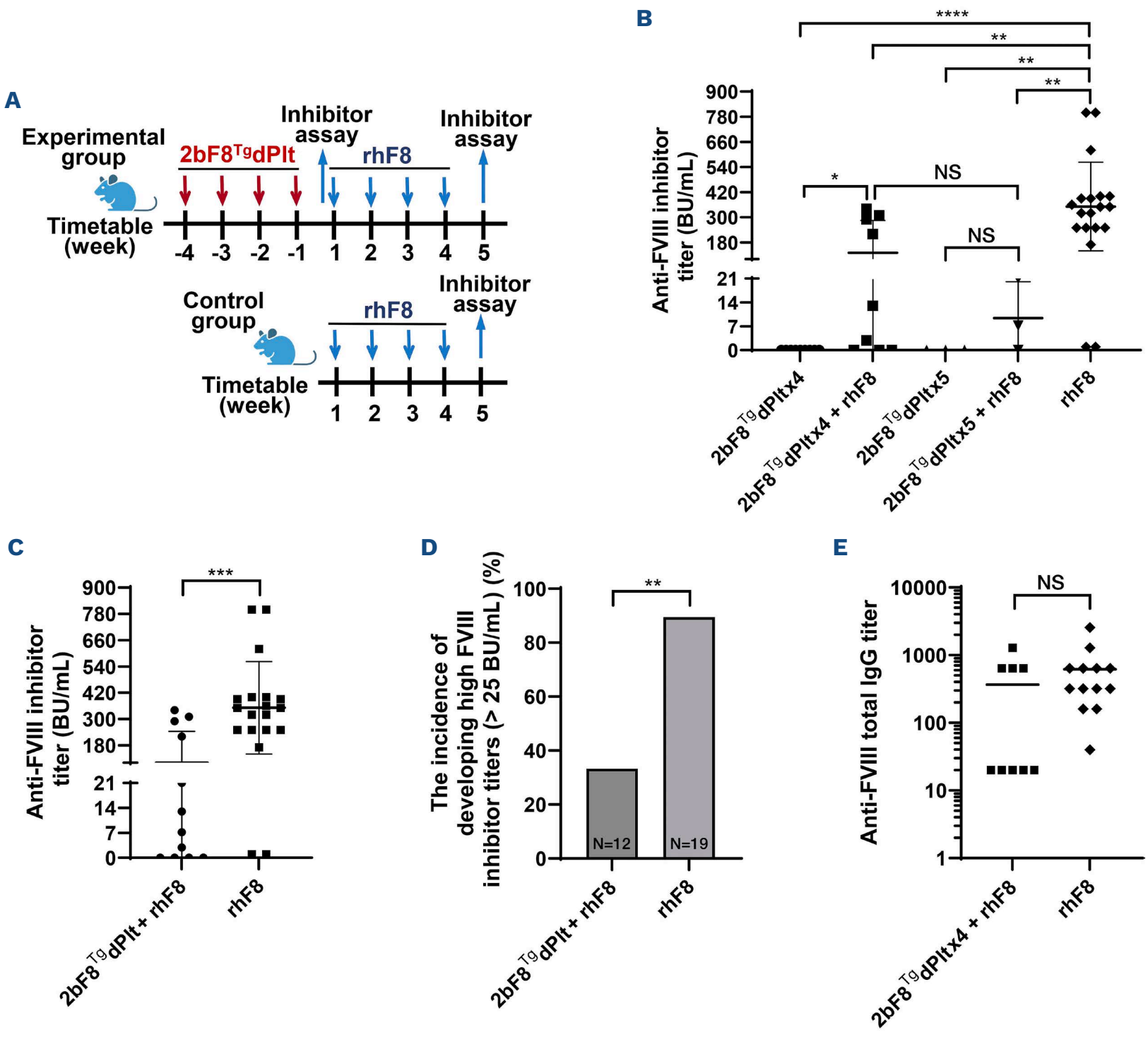


Figure 4. The immune tolerance induction effect of desialylated platelets containing FVIII on FVIII immune responses in FVIII^{null} (B6/129) mice. Blood samples were collected from 2bF8 transgenic (2BF8^{Tg}) mice, and platelets (Plt) were isolated. These Plt were desialylated using our optimized protocol (10 mU/mL of neuraminidase with 5x10^8 Plt/mL, incubated at 37°C for 0.5 hours, then at room temperature for 4.5 hours). The desialylated Plt (2bF8^{Tg}dPlt) 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}dPlt 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}dPlt + rhF8 group presented in (C and D) included data from mice with 4x and 5x dPlt pre-sensitizations. (E) Anti-FVIII total IgG titers. The 2bF8^{Tg}dPlt + rhF8 group presented in (E) included data from mice with 4x dPlt pre-sensitizations. Data are summarized from 4 trials of 2bF8^{Tg}dPlt infusion studies **P<0.01; ***P<0.001; ****P<0.0001; "NS" indicates no statistically significant difference between the 2 groups by either one-way ANOVA (B), unpaired Student t test (C and E), or Fisher's exact test (D).

were comparable in animals before and after 2bF8^{Tg}dPlt infusions (*Online Supplementary Figure S4*).

There were no detectable FVIII inhibitors in HA mice after receiving four or five rounds of 2bF8^{Tg}dPlt infusions. After rhF8 immunizations, the FVIII inhibitor titers in FVIII^{null}(B6/129) mice that received 2bF8^{Tg}dPlt transfusions, including both four and five 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 four and five sensitizations (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}dPlt 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 Treg by FACS. We found that the frequency of Treg in peripheral blood significantly increased in FVIII^{null} mice after 2bF8^{Tg}dPlt transfusions but remained comparable after rhF8 immunization (Figure 5A, B). There was no significant difference in outcomes after rhF8 immunization between the groups that received or did not receive 2bF8^{Tg}dPlt pre-sensitizations (Figure 5C). We further performed the T-cell proliferation assay[30] on whole splenocytes isolated from mice 1 week after the last rhF8 immunization. As shown in Figure 5D, E, the daughter CD4$^+$ T cells from splenocytes that were pre-sensitized with 2bF8^{Tg}dPlt 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}dPlt does not elicit FVIII-specific immune responses but induces Treg cell expansion. Pre-sensitization with 2bF8^{Tg}dPlt 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}Plt and rhF8 co-infusions. In comparison, FVIII^{null}(B6/129) mice died after three to four doses of 2bF8^{Tg}Plt and rhF8 co-infusions, although they all survived with more than four doses of a single infusion of either 2bF8^{Tg}Plt 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}Plt. The inhibitor titer in the 2bF8^{Tg}Plt and rhF8 co-infused (2bF8^{Tg}Plt + rhF8) group was 17.5±28.6 BU/mL, which was significantly lower than in the rhF8 group (Figure 6A-C). The Plt number counts were comparable in animals before and after four doses of 2bF8^{Tg}Plt and rhF8 co-infusions (*Online Supplementary Figure S5*). The frequency of Treg cells in the peripheral blood and spleen in the 2bF8^{Tg}Plt and rhF8 co-infused group was comparable to the rhF8 group (*Online Supplementary Figure S6*).

No FVIII inhibitors were detected in animals that received 2bF8^{Tg}Plt transfusion (Figure 6C). The titer of anti-FVIII total IgG in the 2bF8^{Tg}Plt 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 WtPlt, 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}Plt (2bF8^{Tg}dPlt) 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}dPlt 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 Plt containing FVIII can suppress anti-FVIII immune responses, and pre-sensitized HA mice treated with desialylated Plt containing FVIII can modulate FVIII antibody development.

Discussion

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

Previous studies show Plt 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 Plt also contributes to immune regulation. One notable observation in our study was that HA mice co-infused with FVIII-containing Plt and rhF8 exhibited significantly lower FVIII inhibitor titers than those receiving rhF8 alone. This suggests that Plt-delivered FVIII, along with its Plt contents, has an immunomodulatory effect during antigen exposure to rhF8. The exact mechanism by which co-infusion of FVIII-engineered Plt modulates FVIII immune responses remains unclear. The attenuated immune response is more plausibly explained by antigen delivery in a Plt-derived, TGF-β1-rich tolerogenic milieu rather than by physical sequestration of rhF8. Plt can directly interact

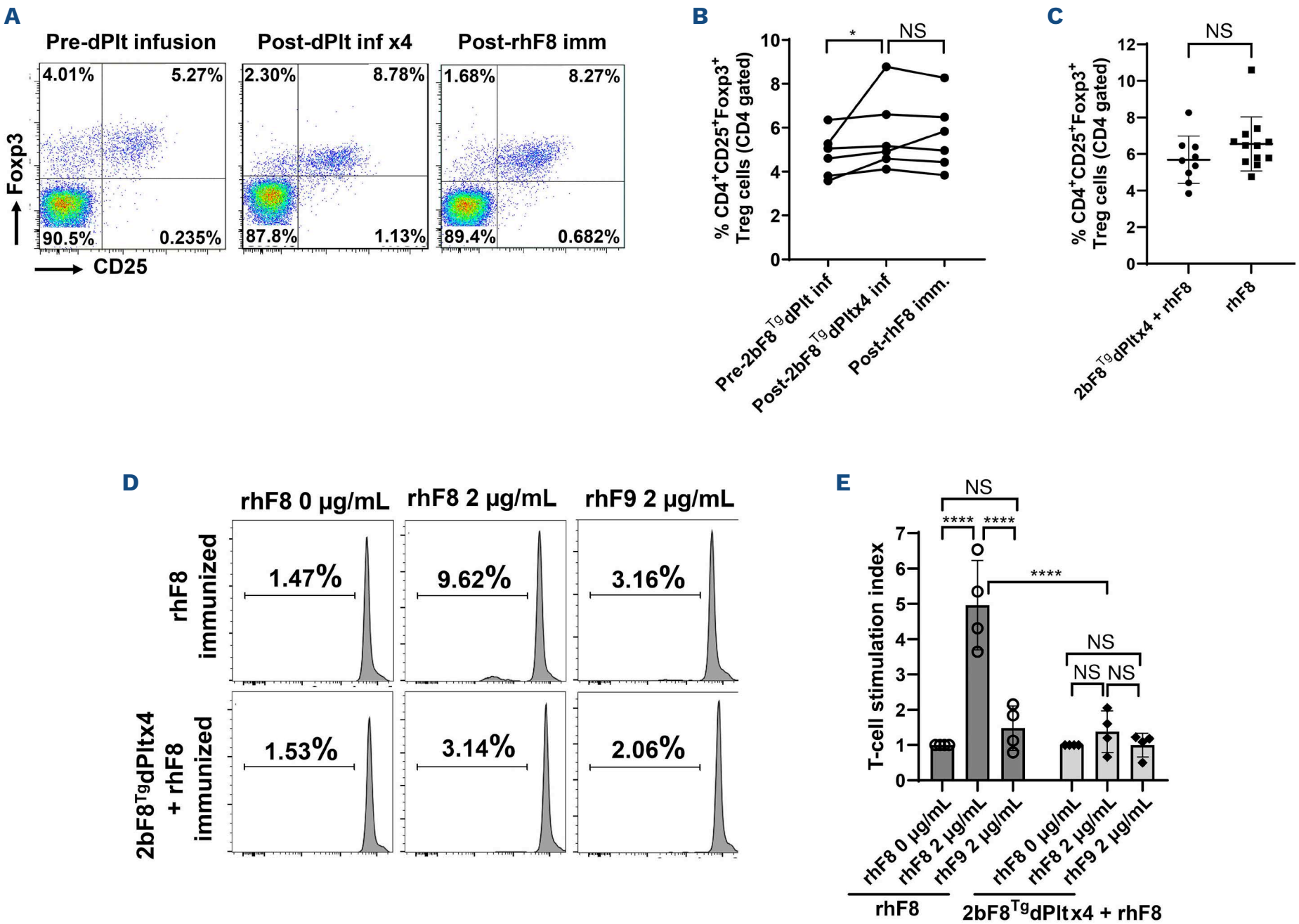


Figure 5. Desialylated platelet infusion expands T-regulatory cells and suppresses T-cell proliferation. For T regulatory (Treg) cell analysis, blood samples were collected via retro-orbital bleeds with 3.8% sodium citrate as an anticoagulant. Leukocytes were stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry. For the T-cell proliferation assay, splenocytes were isolated from animals 1 week after the last recombinant human FVIII (rhF8) immunization (imm.). Whole splenocytes were labeled with the fluorescent dye CellTrace™ 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 37°C 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 T-cell receptor (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 transgenic disialylated platelets (2BF8^{Tg}dPlt) transfusion and rhF8 immunization. (B) The percentages of Treg cells in FVIII^{null}(B6/129) mice before and after 2bF8^{Tg}dPlt 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}dPlt 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 2 trials. *P<0.05; **P<0.01; "NS" indicates no statistically significant difference between the 2 groups by one-way non-parametric Friedman test or Paired t test for (B), unpaired Student t test for (C), or two-way ANOVA for (E). inf: infusion.

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 Plt may directly engage splenic antigen-presenting cells (APC) 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]

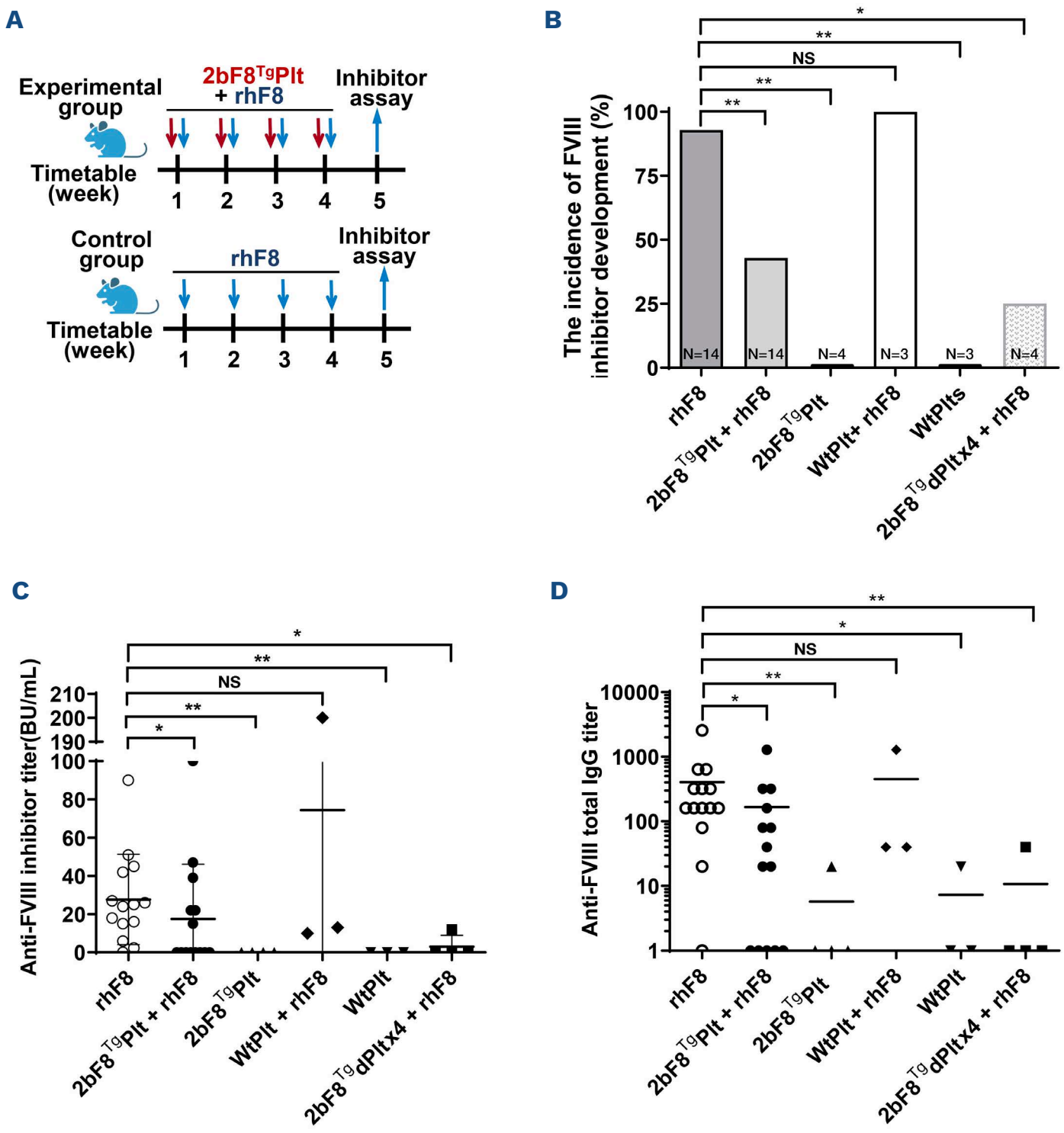


Figure 6. The modulatory effect of platelets containing FVIII on FVIII immune responses in F8^{KO}(B6) mice. Platelets (Plt) were isolated from 2bF8 transgenic (2bF8^{Tg}) or wild-type (Wt) mice. 2bF8^{Tg}Plt or WtPlt were infused into FVIII^{null} mice in a B6 background (F8^{KO}(B6)) along with recombinant human FVIII (rhF8 50 U/kg) via intravenous administration weekly for 4 weeks. The impact of desialylated 2bF8^{Tg}Plt (2bF8^{Tg}dPlt) on FVIII immune responses was also evaluated in this colony of mice, in which 2bF8^{Tg}dPlt 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 enzyme-linked immunsorbent assay. (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 2 groups by the one-way ANOVA.

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 MDSC 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} Plt 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] Plt lysate-induced Treg have been previously shown to exhibit superior stability and suppressive capacity compared to iTreg 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 Plt contents: creating a tolerogenic cytokine environment and presenting the FVIII antigen non-inflammatory, thereby promoting antigen-specific tolerance. Plt 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 Plt with anti-GPIb or IgG, followed by co-infusion with rhF8, did not affect inhibitor titers. This discrepancy might reflect the extent of Plt clearance: systemic anti-GPIb antibodies target all Plt and create a tolerogenic environment, whereas opsonized 2bF8^{Tg}Plt constitute only a small fraction and are cleared gradually *in vivo*. Under these conditions, the tolerogenic signal from a small number of antibody-coated Plt is probably insufficient to counteract the strong immunogenic stimulus from soluble rhF8. Interestingly, repeated co-infusion of rhF8 with 2bF8^{Tg}Plt pre-incubated with anti-GPIb or isotype IgG led to increased peripheral Plt counts after four weekly infusions. A possible explanation is that IgG-opsonized Plt 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 Plt clearance and increases thrombopoiesis, thereby raising Plt counts. Conversely, 2bF8^{Tg}Plt without antibody do not activate Fcγ receptors and do not alter Plt homeostasis.

Our previous study has demonstrated that infusion of intact Plt containing FVIII cannot induce immune tolerance in HA mice, whereas preconditioning animals with sublethal irradiation followed by 2bF8^{Tg}Plt infusion can, an effect attributed to resynchronizing the immune system.[25] In the current study, we demonstrated that repeated transfusion of 2bF8^{Tg}dPlt induced immune tolerance. Mice pre-sensitized with 2bF8^{Tg}dPlt 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 dPlt are rapidly removed by hepatocytes and splenic macrophages, creating an immunosuppressive environment.[18,19] Moreover, infusion of 2bF8^{Tg}dPlt significantly increased circulating Tregs, providing a potential mechanistic link between dPlt 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}dPlt act as tolerogenic vehicles, delivering FVIII antigen to APC in a manner analogous to apoptotic cell therapy. Importantly, our optimized desialylation protocol achieved greater than 95% efficiency without Plt activation, ensuring that the observed effects were due to glycan modification rather than Plt activation artifacts.

Our findings collectively reveal a hierarchy of immune modulation: co-infusion of 2bF8^{Tg}Plt 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}Plt. Acidified Plt lysates elicited more pronounced suppression, likely via direct TGF-β1-mediated immunomodulation. Repeated presensitization with 2bF8^{Tg}dPlt induced immune tolerance, potentially leading to FVIII unresponsiveness. These complementary strategies offer flexibility for different clinical scenarios. For example, co-infusion of FVIII Plt with rhF8 could be used prophylactically at the time of initial FVIII exposure in previously untreated patients to lower the risk of inhibitor formation. Plt lysates could be used as an adjunct to ITI protocols or in patients in whom live Plt 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 Plt 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 Plt 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 dPlt 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 Plt and Plt-derived products are not only hemostatic agents but also potent immune modulators. By leveraging Plt' 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 HA care: the development of FVIII inhibitors.

Disclosures

No conflicts of interest to disclose.

Contributions

YC performed the experiments, analyzed data, and wrote the manuscript. FX performed the experiments and analyzed data. SK performed the experiments and analyzed data. JAS analyzed data and edited the manuscript. WJ discussed and interpreted part of the data. HN provided conceptual input on the platelet desialylation study and edited the manuscript. QS designed and supervised the study, analyzed the data, and wrote the manuscript.

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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 Appendix. For additional information, please contact the corresponding authors.

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