Supplementary Data

Flow cytometry analysis

The spleens or lymphoid nodes (LNs) were isolated from rhF8-immunized FVIII^{null} mice. Single-cell suspensions of spleen or LNs were prepared by standard gentle mechanical disruption. The fluorochrome-conjugated antibodies and clone information were as follows: anti-B220 (RA3-6B2), anti-CD4 (RM4–5 and GK1.5), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-ICOS (C398.4A), anti-CD25 (PC61.5), anti-PD-1 (J43), anti-CXCR5 (SPRCL5), anti-CD40L (MRI), anti-Fas (15A7), anti-GL7 (GL7), anti-Ki-67 (B56), anti-CTLA4 (VC10-4B9), anti-BCL-6 (BCL-DWN), anti-Tbet (eBio4B10), rat IgG2a κ, and mouse IgG1 isotype control antibodies were from eBiosciences (San Diego, CA). Anti-CD3 (145-2C11) and anti-CD4 (GK1.5) were from BD Biosciences (Sparks, MD). Anti-GATA-3 (16E10A23) and mouse IgG2b κ isotype were from BioLegend (San Diego, CA). For detection of Ki-67, Bcl6, Tbet, GATA-3, CTLA4 and Foxp3, surface-stained cells were fixed and permeabilized with the Foxp3/TF Staining Buffer Set (eBiosciences), followed by incubation with corresponding fluorochrome-conjugated antibodies. Cells were analyzed by LSRII or LSRFortessa™ X-20 (BD Biosciences, Franklin Lakes, NJ) flow cytometer and data were analyzed using the FlowJo software (Tree Star, Inc.). Doublets were gated out during FlowJo analysis to exclude possible B-T cell conjugates.

T-cell proliferation assay

T-cell proliferation assays were performed following the procedures described in our previous reports.[32;33] Briefly, splenocytes were isolated from rhF8-immunized FVIII^{null} mice and labeled with the cell proliferation tracer CellTrace Violet (Life Technologies, Carlsbad, CA) following

the protocol provided by the manufacturer. Cells were cultured in completed RPMI-1640 media containing rhF8 (0, 1, or 10 U/mL) or unrelated protein recombinant human FIX (rhF9) (0.4 U/ml, which equates to 2 μg/ml, the same protein amount as 10 U/ml of FVIII) for 96 hours. Cells were harvested, stained for viability, surface markers CD4, CD3, CD19, and CXCR5, and intracellular markers BCL6, Tbet, and GATA3. Isotype antibodies were used as controls in parallel. Samples were analyzed by flow cytometry for CellTrace Violet and cell marker expression**.**

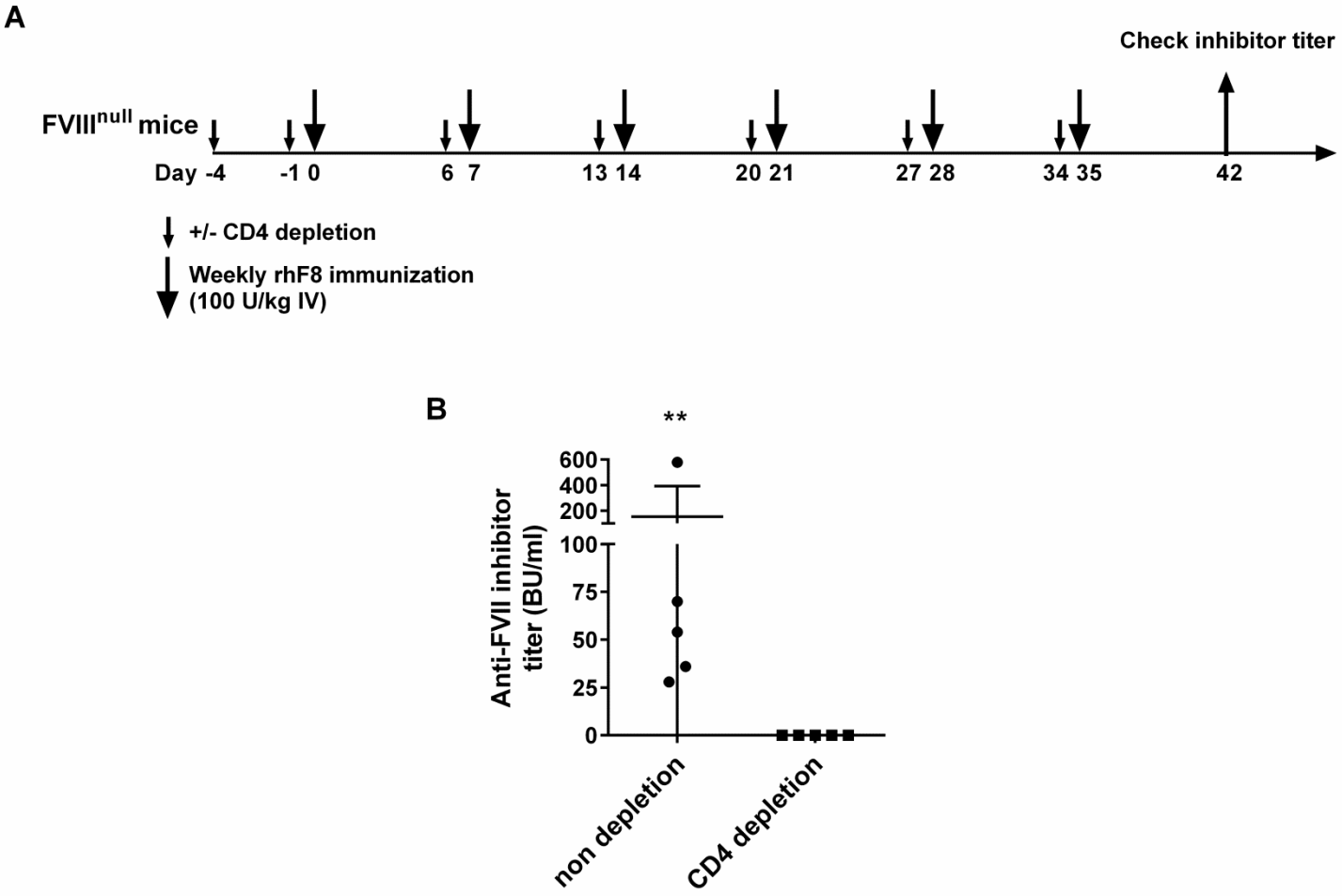
Supplementary figure legend

Supplementary Figure S1. Depletion of CD4 cells during F8 immunization prevents anti-F8 inhibitor induction. (**A**) Experimental scheme for the anti-CD4 treatment and FVIII immunization of FVIII deficient mice. CD4 T cells were depleted by the administration of anti-CD4 antibody GK1.5 on days -4 and -1 before the first immunization and one day before every other immunization. FVIII immunization was given by intravenous injection once per week. (**B**) Inhibitor titers. Seven days after the 6th injection with rhF8. Plasma was collected and anti-FVIII inhibitor titers were determined by chromogenic-based Bethesda assay. $^{**}P < .01$.

Supplementary Figure S2. Depletion of CD4 during FVIII immunization prevents anti-F8 inhibitor induction in WT mice. (A) Experimental scheme for the anti-CD4 treatment and FVIII immunization of wild-type mice. CD4 T cells were depleted by the administration of anti-CD4 antibody GK1.5 on days -4 and -1 before the first immunization and one day before every other immunization. FVIII immunization (rhfF8, 200 U/kg) was given by intravenous injection once per week. (**B**) One week after the 4th injection with FVIII, plasmas was collected for

Bethesda assay to determine anti-FVIII inhibitor titers. **$P < .01$.

Supplemental Figure S1



Supplemental Figure S2