Supplemental Material and Methods

Antibodies and reagents

The following rat anti-mouse monoclonal antibodies (MoAbs) directly conjugated with fluorophore purchased from eBioscience (San Diego, CA) were used in our studies for flow cytometry analysis: CD45.1, CD45.2, CD4, CD8, B220, CD25, and Foxp3. Anti-mouse CD41 MoAb directly conjugated with phycoerythrin was purchased from Santa Cruz Biotech (Santa Cruz, CA). Mouse BD Fc Block was purchased from BD Pharmingen (Franklin Lakes, NJ). Biotinylated anti-CD45.2, CD117, CD4, and CD8 were purchased from BioLegend (San Diego, CA). Streptavidin-SAP was purchased from Advanced Targeting Systems (San Diego, CA). The EasySep™ Mouse SCA1 Positive Selection Kit was purchased from StemCell Technologies Inc. (Cambridge, MA). The QIAamp DNA Blood Mini Kit was purchased from QIAGEN (Germantown, MD). GoTaq® Green Master Mix was purchased from Promega (Madison, WI). Coatest VIII:C/4 Kit was purchased from Diapharma (Franklin, OH). Recombinant human B-domain deleted FVIII (rhF8, Xyntha) was purchased from Pfizer (New York, NY).

Peripheral blood analysis

Blood samples were collected as previously described[1;2] for blood counts and chimerism analysis. Whole blood counts were performed using the Scil Vet ABC Plus blood counter (Scil Animal Care Company, Gurnee, IL). Leukocytes were isolated and stained for cell markers CD45.1, CD45.2, CD4, CD8, B220, CD25, Foxp3 and analyzed by flow cytometry as described in our previous report.[3] Myeloid cells were calculated using 100% of leukocytes minus the percentage of CD4, CD8, and B cells. Platelets were stained for cell marker CD41 as described in our previous report.[4;5] Peripheral blood cell chimerism was determined by flow cytometry

analysis of cell surface markers CD45.1 and CD45.2 in a setting in which the congenic markers can be used to track donor- and recipient-derived cells. For transplants using GFP^{Tg}/CD45.2 donor cells, chimerism was determined using the GFP^+ marker within the $CD45^+$ gate for leukocytes or the $GPIIb^+$ gate for platelets.

PCR and quantitative real-time PCR (qPCR)

DNA was purified from peripheral leukocytes using the QIAamp DNA Blood Mini Kit. A 153-bp fragment from the 2bF8 cassette was amplified as described in our previous report[2] using primers 5-ATCGCTAAGCCAAGGCCACCCT-3 and 5-GCTCCCTCAGAAGCTTTCCAGTAGGA-3. To confirm the $FVIII^{null}$ background, primers (5'-GAGAACCTGCGTGCAATCCATCTT-3' and 5'-GAGCAAATTCCTGTACTGAC-3') amplifying a 350-bp fragment of the disrupted exon 17 of murine FVIII were used. Another set of primers, 5'-GCAAGGGAAGTGATATCACT-3' and 5'-TCCTGTACTGACACTTGTCTC-3', was used to amplify a 233-bp fragment from the undisrupted wild-type (WT) FVIII gene as a control to confirm the exon 17 disruption. DNA from a known 2bF8 transgenic mouse[6] served as a 2bF8-positive control, and water was used as a negative control.

The average copy number of 2bF8 proviral DNA per cell in recipients was determined by quantitative real-time PCR (qPCR) as described in our previous report.[2] Briefly, leukocyte-derived genomic DNA was analyzed for quantification of the 2bF8 expression cassette sequence, with normalization to the murine *Apo B* gene with IDT PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Inc. Coralville, IA). Triplicates were performed for all real-time PCR reactions using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR system

(Thermo Fisher Scientific, Waltham, MA). DNAs from 2bF8^{Tg},[2;6] FVIII^{null} and WT mice was used as controls.

Phenotypic correction assessment

A tail bleeding test and a joint injury model were used to assess the bleeding phenotype in recipients at least 4 months after 2bF8LV gene therapy. We used a 6-hour tail bleeding test to grade the phenotypic correction in 2bF8LV-transduced recipients as described in our previous reports.[5;7-9] For the joint injury model, we used a needle-induced knee joint injury as previously described.[10-13] Briefly, hair was removed from the hind limbs and joint injury was induced (right knee) by transcutaneous insertion of a G30 needle through the patellar ligament using aseptic technique. The uninjured left knee served as an intra-animal control. Knee joints were evaluated by visual assessment of bleeding /bruising. The external diameter across the joint from lateral to medial was measured before and 24 hours after the injury using a digital caliper micrometer. Three replicate measurements of each joint were recorded, and data were averaged. Buprenorphine was administered for post-procedural pain relief. The measurement of baseline diameter of each joint was defined as 1. FVIII^{null} and WT mice were tested in parallel as controls.

FVIII immune responses study

Plasmas were collected from animals and the titers of anti-FVIII inhibitory antibodies (inhibitors) at different time points after gene therapy were determined by a modified Bethesda assay as previously described.[1] After at least 24 weeks of gene therapy, animals were challenged with rhF8 at a dose of either 100 U/kg or 200 U/kg weekly for 4 weeks by intravenous

administration and plasmas were collected one week after the last immunization for Bethesda assay to determine inhibitor titers. $FVIII^{null}$ mice were used as a control in parallel.

Statistical analysis

Data are presented as the mean ± SD. Statistical comparisons of two experimental groups were evaluated by two-tail Student's *t* test or the Mann-Whitney test depending on whether data distribution passes the Normality test. The Fisher Exact test was used to compare the percentage of animals whose tail bleeding stopped within 6 hours after tail tip transection and the incidence of anti-FVIII inhibitor development and. The ANOVA followed by the Dunnett's multiple comparisons test was used to determine whether there were statistically significant differences between the means of three or more groups. The two-way ANOVA followed by the Tukey's multiple comparisons test was used for comparison between groups at different time points throughout the studies. Statistical analysis was performed using Software SigmaPlot 14.0 (Systat Software, Inc. San Jose, CA) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA). A value of $P < 0.05$ was considered statistically significant.

Reference List

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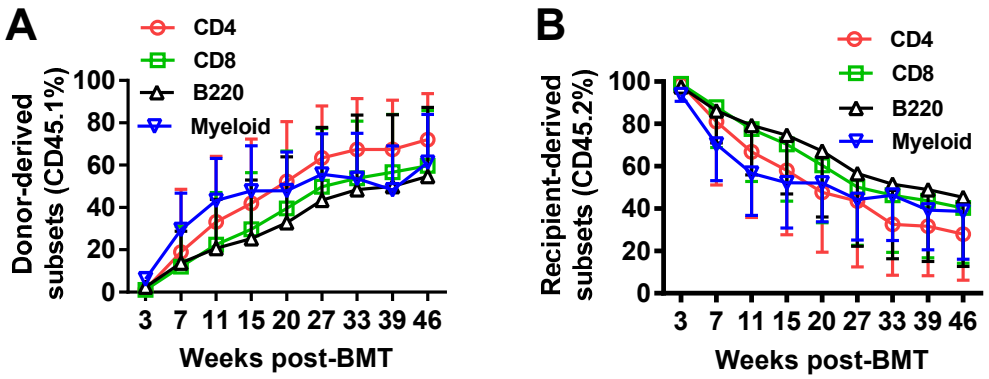
Supplemental Figure Legend

Supplemental Figure 1. Flow cytometry analysis of chimerism in each subset of cells in 2bF8LV-transduced recipients under (CD45.2+CD117)-ADC preconditioning. Blood samples were collected from recipients monthly. Leukocytes were isolated and stained with CD45.1, CD45.2, CD4, CD8, and B220 antibodies and analyzed by flow cytometry. (**A**)

Percentage of donor-derived cells in each subset. (**B**) Percentage of recipient-derived cells in each subset.

Supplemental Figure 2. Chimerism in 2bF8LV-transduced recipients under various preconditioning regimens. Blood samples were collected at 11 weeks after transplantation. Leukocytes were isolated and stained for CD45.1 (donor-derived cell marker), CD45.2 (recipient-derived cell marker), CD4, CD8, and B220. (**A**) Representative dot plots from flow cytometry analysis. (**B**) Summarized data from each group of animals. Data are presented as mean ± SD. The statistical differences between the groups were analyzed using the two-way ANOVA followed by the Tukey's multiple comparisons test. * $P < .05$; ** $P < .01$; *** $P < .001$; and **** $P < .0001$.

Supplemental Figure 1



Supplemental Figure 2