SUPPLEMENTARY APPENDIX

Correction of bleeding in experimental severe hemophilia A by systemic delivery of factor VIII-encoding mRNA

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Supplements

Supplemental method

Quantification of FVIII antigen (FVIII:Ag)

96-well ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated with a mouse monoclonal anti-C2 domain IgG (ESH-8, Sekisui Diagnostics, Kings Hill, Kent, UK) at 1 μg/mL in bicarbonate buffer pH 9.5 for 1 hour at 37°C. Wells were saturated using 3% bovine serum albumin (BSA) in 20 mM Hepes pH 7.4, 150 mM NaCl, 0.05% tween 20. Serial dilutions of plasma or cell culture supernatants in 20 mM Hepes pH 7.4, 600 mM NaCl, 0.05% tween 20 were incubated for 1 hour at 37°C. FVIII was detected using a mouse monoclonal biotinylated anti-A2 domain antibody (GMA 8015, Green Mountain Antibodies, Burlington, VT, USA) and revealed by streptavidin-horse radish peroxidase (R&D Systems, Minneapolis, MN, USA) and its 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (eBioscience, Thermo Fisher Scientific). For quantitation of the FVIII light chain in mouse plasma (which represents both the active and inactive levels of FVIII), plates were coated with the human anti-C2 monoclonal IgG BO2C11 (a kind gift from Prof Saint-Remy and Jacquemin, KUL, Leuven, Belgium) and the light chain was recognized with biotinylated EHS-8. Recombinant human BDD-FVIII (Refacto®, Pfizer) was used as a standard.

Quantification of FVIII activity (FVIII:C)

FVIII:C was measured in culture supernatant or in mouse plasma using a FVIII chromogenic Assay (Siemens, Munich, Germany) and using pooled human plasma as a standard. Apparent specific activities were calculated by dividing the FVIII:C by FVIII:Ag for each individual plasma/supernatant.

Bleeding time

FVIII-deficient mice were injected intravenously with 3 µg of FVIII-encoding mRNA, 1.2 or 3 IU of rFVIII or PBS as a negative control. Mice were anesthetized with Ketamine/Xylazine (100 mg/kg and 10 mg/kg, respectively), and 3 mm of the distal tail were cut using a scalpel. The amputated tail was immersed immediately in physiologic saline solution at 37°C, and blood was collected for 20 min. The blood was then centrifuged at 3000 g for 15 min and red blood cells pellets were lysed in water for 15 min. The absorbance of released hemoglobin was measured at 416 nm. The corresponding volume of blood lost was calculated using a standard curve prepared by lysing red blood cells prepared from known volumes of mouse blood.

Titration of anti-FVIII IgG

96-well ELISA plates (Nunc Maxisorp) were coated with recombinant FVIII (Recombinate®, Baxter, Vienna, Austria) at 2 µg/mL in bicarbonate buffer pH 9.5 for 1 hour at 37°C and blocked with PBS-3% BSA. Serial plasma dilutions were then incubated for 1 hour at 37°C. Bound IgG were revealed using a goat horse radish peroxidase-coupled polyclonal anti-mouse IgG (SouthernBiotech, Birmingham, AL, USA) and the o-Phenylenediamine Dihydrochloride (OPD) substrate. The mouse monoclonal IgG mAb6 specific for the heavy chain of FVIII (a gift from Prof. J.M. Saint-Remy, KUL, Belgium) was used as a standard.

Titration of FVIII inhibitors

Serial dilutions of mouse plasma were incubated volume to volume with a standard pool of human plasma (Siemens) for 2 hours at 37°C. The residual pro-coagulant FVIII activity was measured using the FVIII Chromogenic assay (Siemens). Bethesda titers, expressed in BU/mL, are defined as the reciprocal of the dilution of plasma that produces 50% residual FVIII activity.

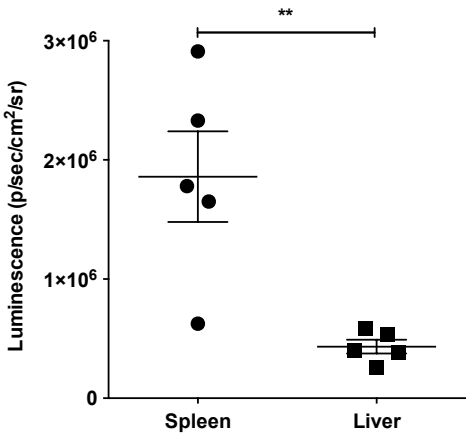
Activation of human monocyte-derived dendritic cells

Monocytes were isolated from the blood of healthy donors using anti-CD14+ magnetic microbeads (Miltenyi Biotec, Paris, France). Monocytes were incubated in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with GM-CSF (1000 IU/10^6 cells) and IL-4 (500 IU/10^6 cells) (Miltenyi Biotec) for five days to generate immature monocyte-derived dendritic cells (MODCs). The immature status was confirmed by flow cytometry (LSR II, BD Biosciences, Le Pont au Claix, France) with a CD1a, CD14, CD80, CD86, CD83, CD40 and HLA-DR staining. MODCs ($0.2x10^6$ cells) were plated in AIM V medium (Invitrogen) and transfected with TransIT alone, 1 µg mRNA alone or 1 µg mRNA formulated in TransIT, as described in "*In vitro* transfection". As controls, MODCs were incubated alone or in the presence of 1 ng/mL LPS or 1 µg/mL Poly:IC. After 8 and 24 hr, TNF-alpha and IL-6 were detected in culture supernatant, using the TNF-alpha and IL-6 DuoSet ELISA (R&D Systems Europe, Lille, France).

Luciferase detection

Wild-type 8 week-old Balb/c males (Janvier, Saint-Berthevin, France) were injected intravenously with 5 µg of luciferase-encoding mRNA formulated in TransIT®, and 24 hours later with 225 mg/kg luciferin. After 10 min, luminescence was detected using an In Vivo Imaging System (Lumina II, PerkinElmer, Waltham, MA, USA). Images were acquired and analyzed by Living Image® 4.4 software (PerkinElmer).

Supplementary Figures



Supplemental Figure 1. Production of Luciferase in Balb/c mice treated with luciferase-encoding mRNA. Five Balb/c mice were injected intravenously with 5 µg of luciferase-encoding mRNA formulated in TransIT®. The expression of luciferase was measured 24 hours later, following the intravenous injection of luciferin. Mice were dissected and the luciferase activity was measured in the spleen and liver. Luminescence was quantified and is expressed as p/sec/cm^2/sr, where p stands for photon and sr for steradian. Individual mice are depicted as single dots and horizontal bars depict means±SEM. Statistical differences were assessed using the double-sided Mann-Whitney test (**: $p<0.01$).