SUPPLEMENTARY APPENDIX

The C1 and C2 domains of blood coagulation factor VIII mediate its endocytosis by dendritic cells

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Supplemental Methods

Cloning of cDNA encoding B domain– deleted FVIII mutants

The R2215A, R2220A, R2215A-R2220A, R2090A-K2092A-F2093A and Y1680C mutations were generated by splicing-by-overlap extension mutagenesis (1). The primer set used to construct these mutants as well as the restriction sites used for cloning into the mammalian expression vector ReNeo are listed below. The template used for the PCR reactions was wild-type B domain-deleted FVIII named HSQ in the ReNeo vector (gift from Dr P Lollar, Emory University, Atlanta, GA, USA).

Primers for R2215A:

Forward primer: 5'- ATGGGCTGTGATTTAAATAGTTGCAGCAT -3'

Reverse primer: 5'- TGCTGCAGCGGCCGCCCTCAGTAGAGGTCCTGT -3'

Internal primers: 5'- CCTCCAAGGGGCCAGTAATGCCTGGAGACC -3'

5'- GGTCTCCAGGCATTACTGGCCCCTTGGAGG -3'

Restriction enzymes for ligation to ReNeo: SwaI/NotI

Primers for R2220A:

Forward primer: 5'- ATGGGCTGTGATTTAAATAGTTGCAGCAT -3'

Reverse primer: 5'- TGCTGCAGCGGCCGCCCTCAGTAGAGGTCCTGT -3'

Internal primers: 5'- AGTAATGCCTGGGCCCCTCAGGTG -3'

5'- CACCTGAGGGGCCCAGGCATTACT -3'

Restriction enzymes for ligation to ReNeo: SwaI/NotI

Primers for R2215A-R2220A:

Forward primer: 5'- ATGGGCTGTGATTTAAATAGTTGCAGCAT -3'

Reverse primer: 5'- TGCTGCAGCGGCCGCCCTCAGTAGAGGTCCTGT -3'

Internal primers: 5'- CCTCCAAGGGGCCAGTAATGCCTGGGCCCCTCAGG -3'

5'- CCTGAGGGGCCCAGGCATTACTGGCCCCTTGGAGG -3'

Restriction enzymes for ligation to ReNeo: SwaI/NotI

Primers for R2090A-K2092A-F2093A:

Forward primer: 5'- GACAGTGGGCCCCAAAGCTGGCCAG -3'

Reverse primer: 5'- GCTGCAACTATTTAAATCACAGCCC -3'

Internal primers:

5'- GACCCAGGGTGCCGCACACGCGGCCTCCAGCCTCTACATCTCT -3'

5'- AGAGATGTAGAGGCTGGAGGCCGCGTGTGCGGCACCCTGGGTC -3'

Restriction enzymes for ligation to ReNeo: ApaI/SwaI

Primers for Y1680C:

Forward primer: 5'- GGAAATAACTCGTACGACTCTTCAGTCAG -3'

Reverse primer: 5'- CAGCTTTGGGGCCCACTGTCCATATTG -3'

Internal primers: 5'- GATTTTGACATTTGTGATGAGGATGAAA -3'

5'- TTTCATCCTCATCACAAATGTCAAAATC -3'

Restriction enzymes for ligation to ReNeo: BsiWI/ApaI

Expression of wild-type and mutated FVIII by BHK-M cells

BHK-M cells (gift from Dr P Lollar, Emory University, Atlanta, GA, USA) were transfected using Lipofectamine™ 2000 (Life Technologies, Saint-Aubin, France) according to the manufacturer's instructions. Transfected cells were cultured onto 10 cm² dishes at a density of 1000, 500 and 100 cells/ml in the presence of 10 ml of DMEM/F-12 medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies), 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technologies), and 500 µg/ml geneticin sulphate (Sigma-Aldrich, Saint-Louis, MO, USA). After 10 days, geneticin-resistant clones were picked and harvested in flat-bottom 96-well plates with the same media until confluency, and screened for FVIII production by sandwich ELISA using ESH8 and biotinylated GMA-8015

as capture and detection antibodies, as described in Material and Methods. The highest expressing clones for each FVIII were scaled up to near confluency before switching the medium to serum-free AIM-V medium (Life Technologies) for 24 hr. Collected medium was stored at -80°C before purification.

Purification of wild-type and mutated FVIII

Purification of wild-type and mutated FVIII was performed using i) an affinity chromatography and ii) an anion-exchange chromatography, based on previously described protocols (2). FVIII-containing media were thawed at 37°C, centrifuged at 1000 g for 20 min to remove cell debris and supplemented with 0.05% sodium azide. Pooled medium was loaded onto a VIIISelect column (GE Healthcare, Uppsala, Sweden) and FVIII was eluted in isocratic conditions, with 20 mM HEPES, 1.5 M NaCl, 10 mM $CaCl_2$, 0.01% tween 80, 50% ethylene glycol, pH 7.4. Eluted fraction was then 5-fold diluted in 20 mM HEPES, 5 mM $CaCl_2$, 0.01% tween 80, pH 7.4 and loaded onto a 1 ml anion-exchange HiTrap Resource Q column (GE Healthcare). FVIII was finally eluted using a linear 0.2-1.0 M NaCl gradient. Purified wild-type and mutated FVIII were assessed by chromogenic assay, absorbance at 280 nm and analyzed by 4-12% gradient SDS-PAGE (1 µg per lane) with and without prior activation by bovine thrombin (1 IU, Sigma-Aldrich) and stained with 0.1% silver nitrate as previously described (3).

***In vitro* activation of a FVIII-specific HLA-DRB1*0101-restricted mouse CD4+ T cell hybridoma by murine splenocytes**

Total splenocytes from HLA-DRB1*0101 transgenic SURE-L1 mice were isolated and used as a source of APCs after treatment with 50 µg/ml of mitomcyin C (SIGMA) for 30 min, at 37°C and 3 washes in RPMI-1640. Splenocytes were co-cultured for 18 hr at 37°C in 96

round-bottom plates with the 1G8-A2 FVIII-specific HLA-DRB1*0101-restricted CD4+ T-cell hybridoma (ratio 2:1, 200.000 splenocytes for 100.000 T cells) in the presence of B domain-deleted FVIII (10 nM) alone or pre-incubated with 2 molar excess of ESH8, BO2C11 or BO2C11 Fab fragments. Activation of T cells was measured by IL-2 secretion in the supernatant using BD OptEIA™ mouse IL-2 ELISA set (BD Biosciences). Controls included T cells incubated alone, or incubated with splenocytes in the presence of concanavalin A (2 μg/ml, Sigma-Aldrich) or in the absence of FVIII.

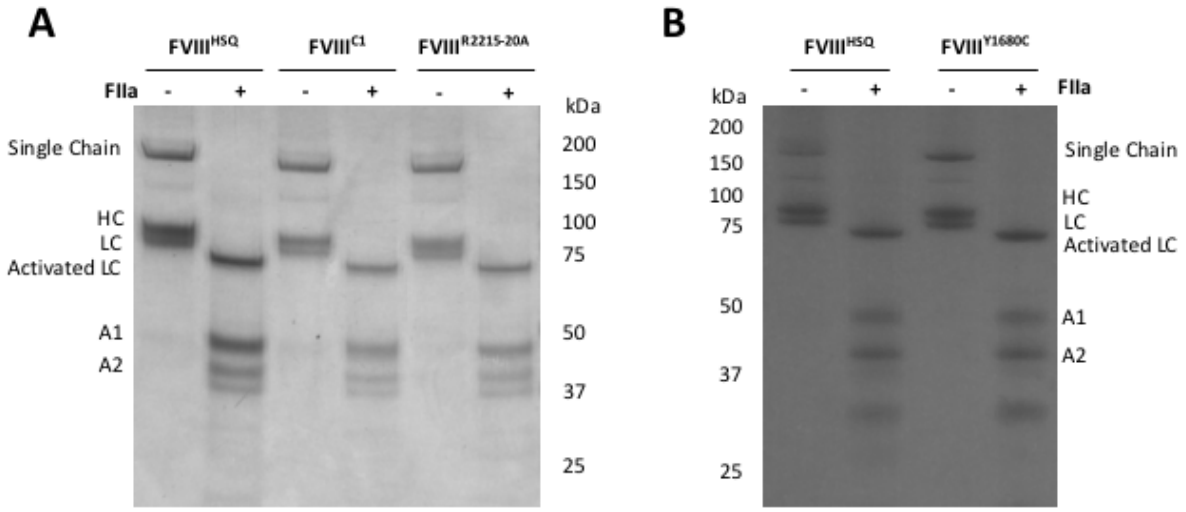
In vitro proliferation of splenocytes

FVIII exon 16 knock-out C57BL/6 mice were injected once a week for 4 weeks with B domain-deleted $FVIII^{Y1680C}$ alone or pre-incubated with BO2C11. One week after the last injection, mice were sacrificed and spleens collected and processed into single-cell suspensions. Red blood cells were eliminated with ammonium-chloride-potassium lysing buffer (Lonza, Verviers, Belgium). Splenocytes were isolated and cultured in round-bottom 96-well plates in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 55 μM 2-mercaptoethanol in the presence of increasing concentrations of $FVIII^{HSQ}$ (0, 0.5, 1, 2 μg/mL) or concanavalin A (ConA, 0, 0.5, 1, 2 μg/mL). After 72 hr, cells proliferation was assayed following the addition of [^{3}H]-thymidine (Perkin Elmer, Villebon-sur-Yvette, France) for 24 hr.

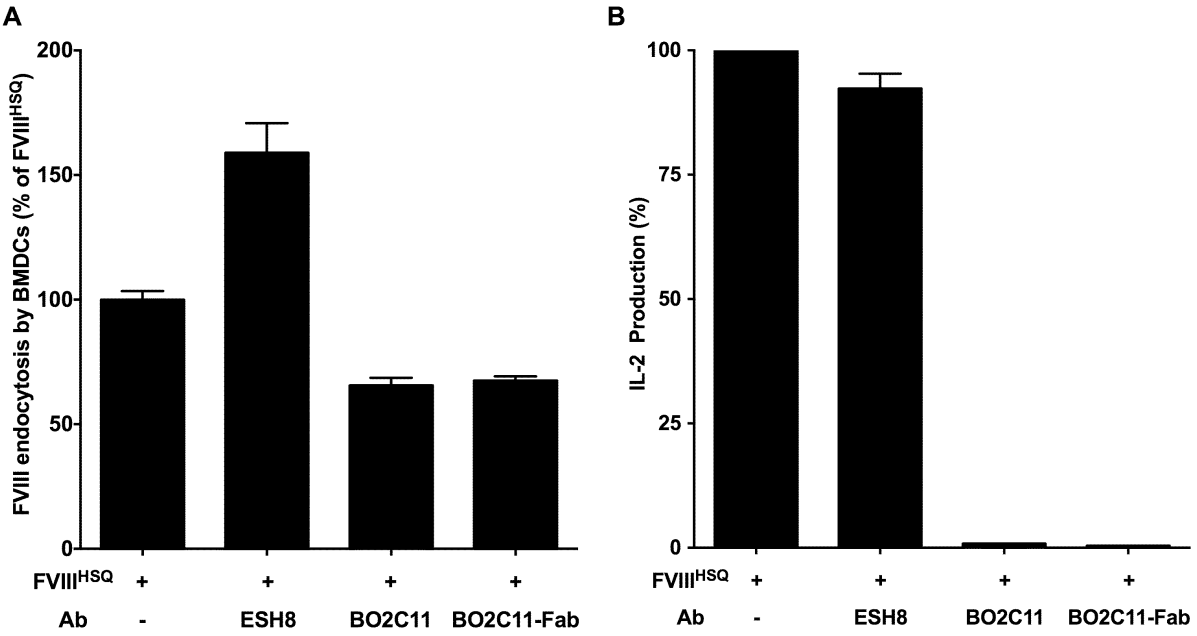
References to supplemental methods

1. Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR. Gene splicing by overlap extension. Methods Enzymol. 1993;217:270-269.
2. Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. High level expression of recombinant porcine coagulation factor VIII. J Biol Chem. 2002 Oct 11;277(41):38345-38349.
3. Morrissey JH. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem. 1981 Nov 1;117(2):307-310.

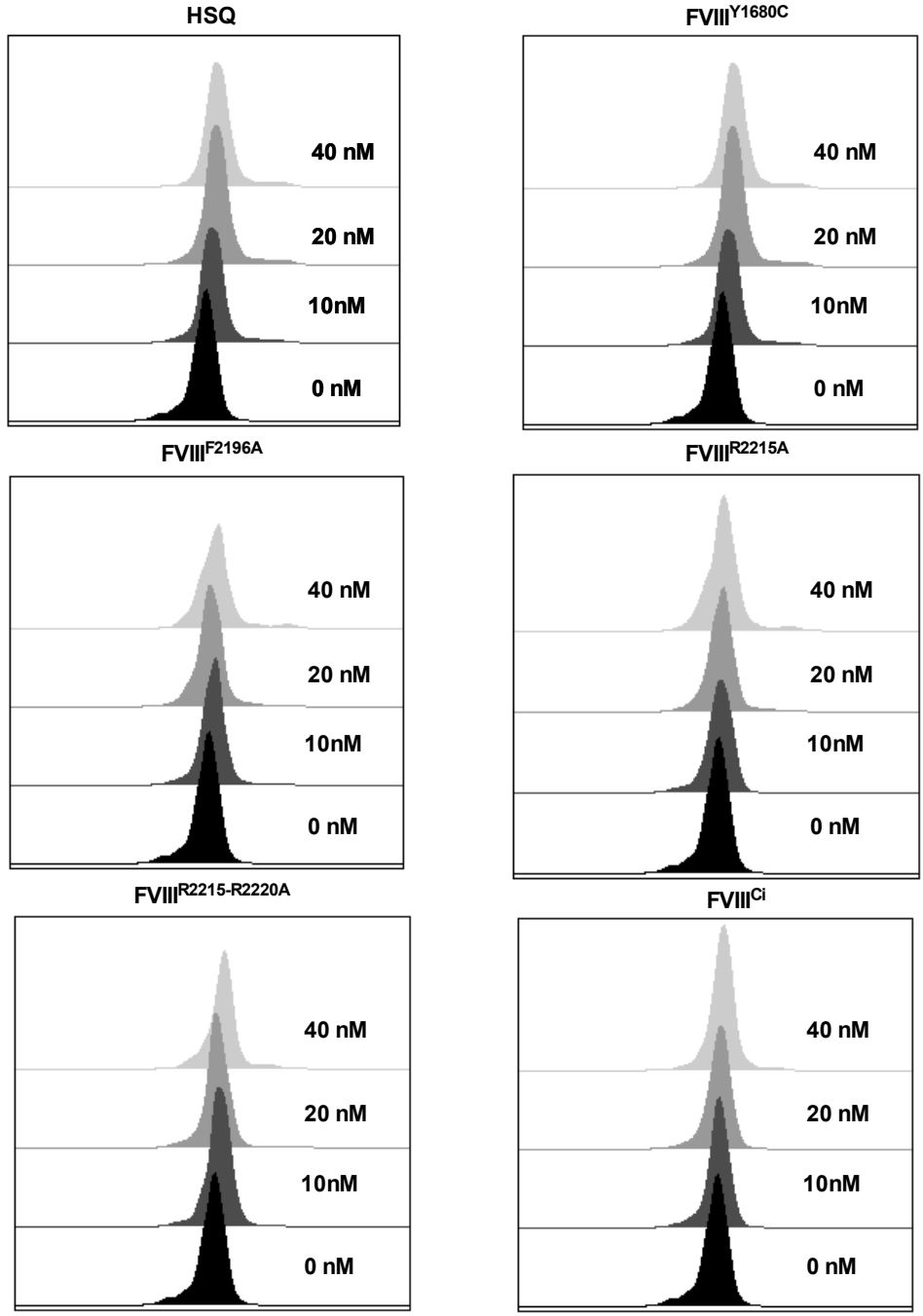
Supplementary figures



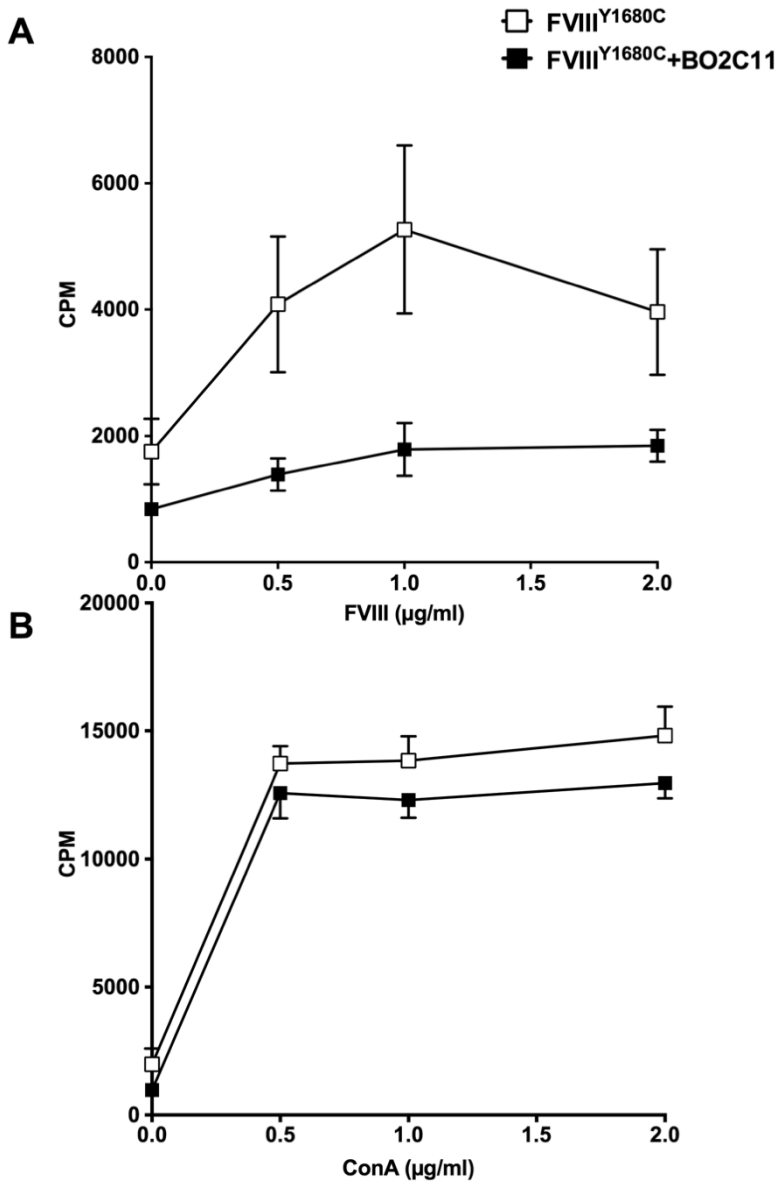
Supplementary Figure 1. SDS-PAGE analysis of the different purified recombinant FVIII. B domain-deleted wild-type and mutated FVIII (1 µg/lane) were incubated with or without 1 IU bovine thrombin (FIIa) for 30 min at 37°C and loaded on 4-12% gradient polyacrylamide gels under non-reducing conditions. Gels were then stained with 0.1% silver nitrate. HC, FVIII heavy chain; LC, FVIII light chain; A1, A1 domain; and A2, A2 domain.



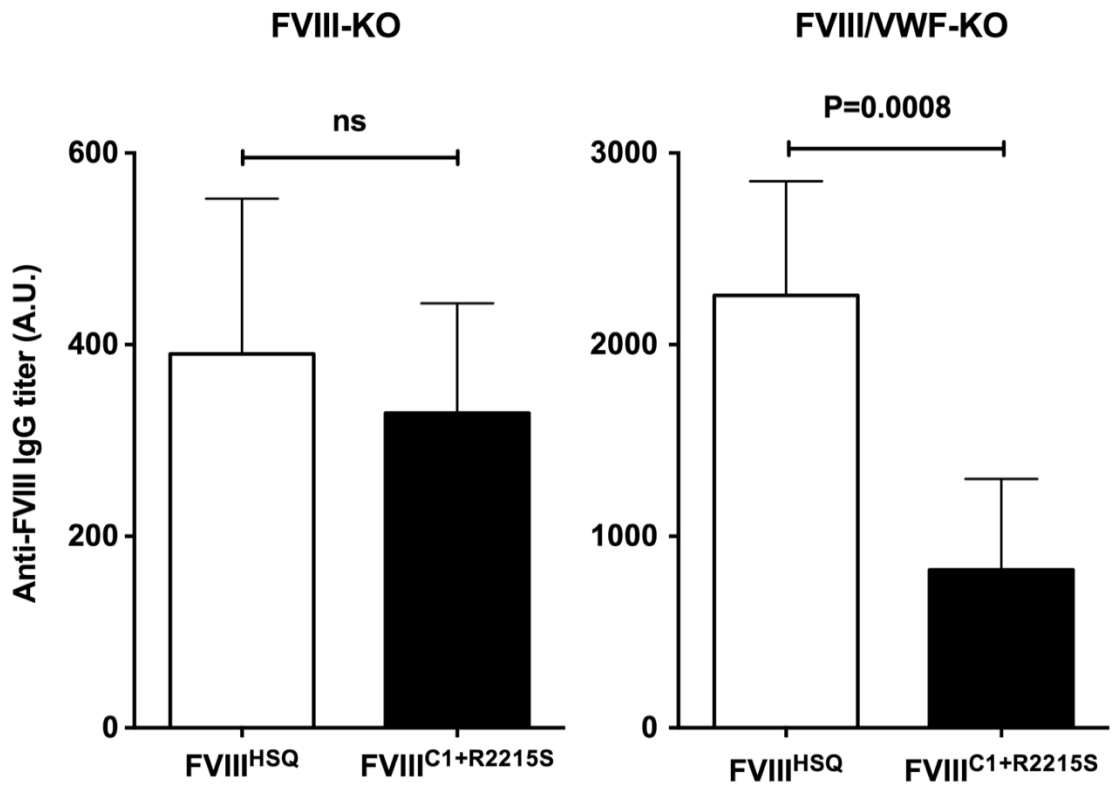
Supplementary Figure 2. The anti-C2 antibody BO2C11 inhibits FVIII presentation *in vitro*. Panel A. B domain-deleted FVIII (20 nM) was pre-incubated with equimolar concentrations of ESH8, BO2C11 or with a 2 molar excess BO2C11 Fab fragments. Uptake by murine bone marrow-derived dendritic cells (BMDCs) was analyzed by fluorescence-activated cell sorting (FACS). Results are expressed as the percentage of median fluorescence intensity (MFI), whereby 100% corresponds to MFI obtained with wild-type FVIII ($FVIII^{HSQ}$). Graphs are representative of 5 individual mice (mean±SEM). Panel B. Total splenocytes from HLA-DRB1*0101 transgenic SURE-L1 mice (used as the source of APCs) were cultured for 24 hr in 96 round-bottom plates with the FVIII-specific HLA-DRB1*0101-restricted CD4+ T-cell hybridoma (ratio 1:10, clone 1G8-A2) in the presence of B domain-deleted FVIII or FVIII pre-incubated with ESH8, BO2C11 or with BO2C11 Fab fragments. Activation of T cells was measured by IL-2 secretion in the supernatant by ELISA (BD Biosciences). Data are depicted as % of IL-2 expression versus $FVIII^{HSQ}$ alone. Results are representative of three experiments (means±SEM).



Supplementary Figure 3. FVIII uptake by MoDCs. Increasing concentrations of B domain-deleted wild-type FVIII (HSQ) or mutants $FVIII^{Y1680C}$, $FVIII^{F2196A}$, $FVIII^{R2215A}$, $FVIII^{R2215-20A}$, or $FVIII^{C1}$ were added to MoDCs for 30 min at 37°C. FVIII endocytosis was assessed as described earlier and data analyzed by FACS.



Supplementary Figure 4. Inhibition of the anti-FVIII immune response *in vivo* upon co-incubation of FVIII with BO2C11. FVIII-deficient mice were injected weekly with B domain-deleted FVIII^{Y1680C} incubated alone or in the presence of BO2C11. One week after the fourth treatment, spleens were collected and processed into single-cell suspensions. Red blood cells were removed and splenocytes were cultured in round-bottomed 96-well plates for 48-72 hr in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 55 µM 2-mercaptoethanol in the presence of increasing concentrations of FVIII^{HSQ} (0, 0.5, 1, 2 µg/mL) or concanavalin A (ConA, 0, 0.5, 1, 2 µg/mL). Proliferation was assayed following the addition of [^{3}H]-thymidine. The results are expressed as counts per minute (CPM, mean±SEM).



Supplementary Figure 5. Immunogenicity of FVIII^{HSQ} and FVIII$^{C1+R2215S}$ in FVIII-deficient and in double FVIII/VWF-deficient mice. FVIII-deficient mice and double FVIII/VWF-deficient mice were injected intravenously once weekly for 4 weeks with 0.5 µg of B domain-deleted FVIII^{HSQ} or FVIII$^{C1+R2215}$. One week after the last injection, blood samples were collected. Anti-FVIII IgG titers were measured by ELISA and are expressed as arbitrary units (mean±SEM) using the mouse monoclonal anti-FVIII IgG Mab6 as a standard. Statistical significances were assessed using the two-tailed non-parametric Mann-Whitney U test (ns: not significant). The specific activities, *in vivo* recoveries and capacities to activate the T-cell hybridoma were similar for FVIII^{C1} and FVIII$^{C1+R2215S}$ (not shown).