Complement C3 is a novel modulator of the anti-factor VIII immune response

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List of abbreviations:

ADAMTS-13: A Disintegrin And Metalloprotease with ThromboSpondin type I repeats-13

APCs: antigen-presenting cells

BDD: B domain-deleted

BSA: bovine serum albumin

BU: Bethesda units

ΔC3: C3-depleted serum

FVIII: factor VIII

FB: factor B

FD: factor D

hCVF: humanized cobra venom factor

HLA: human leukocyte antigen

IL-2: interleukine-2

NS: normal serum

HIS: heat-inactivated serum

HRP: horse radish peroxidase

MO-DCs: monocyte-derived dendritic cells

MO-Φ: monocyte-derived macrophages

OPD: o-Phenylenediamine Dihydrochloride substrate

TTP: Thrombotic Thrombocytopenic Purpura

Supplementary Methods

Preparation of the plasmid expressing HC3-1496. This plasmid was prepared in a manner very similar to the method described earlier for the preparation of pMB-HC3-1348 [1]. Initially, two PCR reactions were performed to obtain the human C3 and CVF portions of the coding sequence, the first using pBS-HuC3(2) as a template and HuC3H5-3-F1 (TCTGTGTGGCAGACCCCTTCGAGG) and HuC3H5-4-R1(2) (GAGAAGGCCTGTTCCTTTATCCGGATGGTAGAACCGGGTAC) as primers, and the second using pCVF-FL3Δ as a template and HuC3H5-4-F2(2)

(CCGGTTCTACCATCCGGATAAAGGAACAGGCCTTC) and HuC3H5-3-R2 (CATCCATGACATAGATATCATTACCATCTTG) as primers. Following the PCR reaction, the products were purified using the Qiagen PCR purification kit, and combined in an overlap extension PCR reaction, using the two PCR fragments as templates and HuC3H5-3-F1 and HuC3H5-3-R2 as primers. This PCR product was purified, cut with BstBI and gel purified as described above. It was then ligated into pHC3-1550(-sig) that had been BstBI cut and dephosphorylated with calf-intestinal phosphatase. Orientation of the inserts was determined by EcoRI digestion, and clones with the inserts in the correct orientation were sequenced to ascertain the correct sequence with a lack of PCR-induced mutations. The resulting plasmid was called pHC3-1496. The HC3-1496 coding sequence was amplified by PCR. There were two amplifications, one to produce a fragment coding for HC3-1496 with the native human C3 signal sequence, and one coding for HC3-1496 with a mouse IgG signal sequence. For producing the coding sequence with the native C3 signal, the following primers were used PNAtf: 5'-gcaagcttGCCGCCACCATGGGACCCACCTCAGGTC-3' and Pnatr: 5'-ccgcggccgcTTAAGTAGGGCAGCCAAACTCAGTCAAT-3'. The primers used for producing the HC3-1496 with the mouse IgG signal sequence were Pmsp: 5'-gcaagcttGCCGCCACCATGGAGACCGACACACTGCTGCTGTGGGTGCT GCTGCTGTGGGTCCCCGGCTCCACTGGAAGTCCCATGTACTCTATCATCACC CCCAAC-3' and Pnatr :5'-ccgcggccgcTTAAGTAGGGCAGCCAAACTCAGTCAAT-3'. The resulting PCR products were gel purified and isolated from the gel using the Qiagen QIAquick Gel Extraction Kit, and cut with HindIII and NotI. The fragments were then cloned into pOptiVEC-TOPO that had been cut with the same enzymes. The expression vectors were called pOptiVEC-1496(sig)-3# and pOptiVEC-1496-3# respectively.

Expression and purification of HC3-1496. pOptiVEC-1496(sig)-3# and pOptiVEC-1496-3# were transfected into CHO cells by electroporation. Following transfection, stably transfected cells were selected using either 200nM or 500nM methotrexate in liquid media. High-producing clones were then selected by plating between 2500 and 10,000 cells onto semisolid media containing a fluorescent anti-CVF antibody in petri dishes. Colonies were allowed to grow for 10 days, and colonies producing HC3-1496 were detected, either by detecting fluorescent colonies, or colonies with a ring of immunoprecipitates. Colonies producing high yields of HC3-1496 were selected, and plaque purified. The HC3-1496 protein was purified by a combination of Capto-Q, Butyl FF HiTrap, and Q-HP HiTrap chromatography.

Production and purification of. The cDNA encoding human B domain-deleted (BDD) FVIII ($FVIII^{HSQ}$) was used as a template to generate the R2090A-K2092A-F2093A FVIII mutant ($FVIII^{C1}$) by splicing-by-overlap extension mutagenesis as described.[2] Presence of the mutations was confirmed by standard sequencing analysis. The stable expression of wild-type and mutated FVIII by baby hamster kidney-derived cells, and FVIII purification were performed as described.[3] FVIII concentration was calculated by absorbance at 280 nm using a molar extinction coefficient of 256,300 $M^{-1}cm^{-1}$ and a molecular weight of 165,300 Da. The purity of recombinant FVIII was confirmed by SDS-PAGE with or without digestion by thrombin (not shown). Specific activities were estimated in a one-stage clotting assay and ranged between 4800-9000 IU/mg.

Maturation of MO-DCs in the presence of complement. Human normal serum, heat-inactivated serum or C3-depleted serum were incubated at 10% with 5-day-old immature MO-DCs (2.10^5 cells/100 µl) in RPMI-1640 in the presence of FVIII (35 nM) for 24 hr at 37°C. Cell surface was stained for CD40, CD80, CD83, CD86 and HLA-DR, and dead cells

were discriminated with propidium iodide (5 μg/ml, Sigma-Aldrich). As a positive control, immature MO-DCs were incubated with lipopolysaccharide (LPS, 1 μg/ml, Sigma-Aldrich). MFI was measured by flow cytometry.

***In vivo* half-life of FVIII**. Mice were injected intravenously with human recombinant FVIII (100 μl at 10 nM, Helixate® NexGen) 6 hours after complement depletion. Blood was collected using heparinized capillaries at 10, 25, 55, 115, 235, 355, 385 and 475 min. FVIII:Ag in plasma was measured by sandwich ELISA, using a mouse monoclonal anti-C2 domain IgG (ESH-8, Sekisui Diagnostics, Kings Hill, United Kingdom) to capture FVIII. Bound FVIII was revealed using a biotinylated monoclonal mouse anti-human FVIII A2 domain antibody GMA-8015 (Green Mountain Antibodies, Burlington, VT, USA), streptavidin-HRP and the OPD substrate. Experimental data were fitted with a one-phase exponential decay equation using GraphPad Prism (version 6).

References

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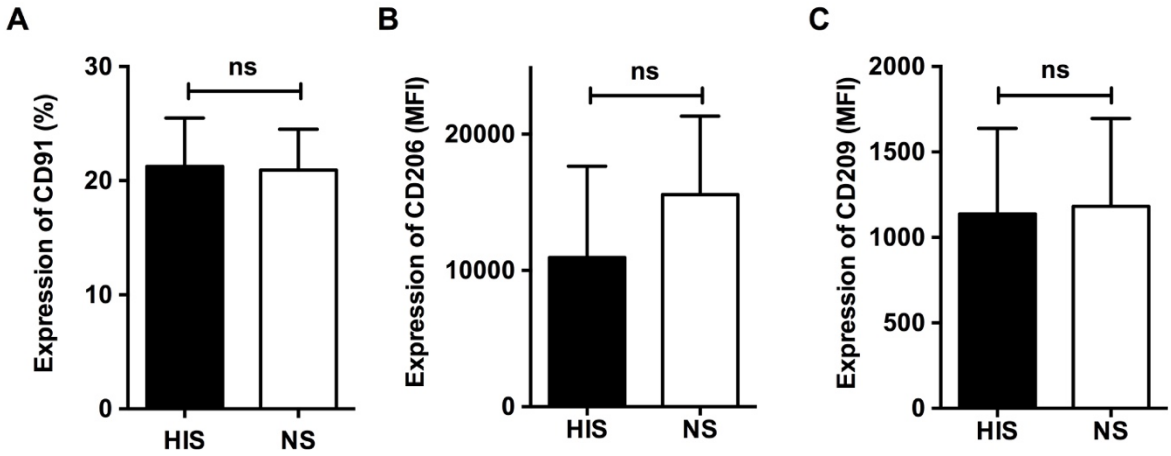
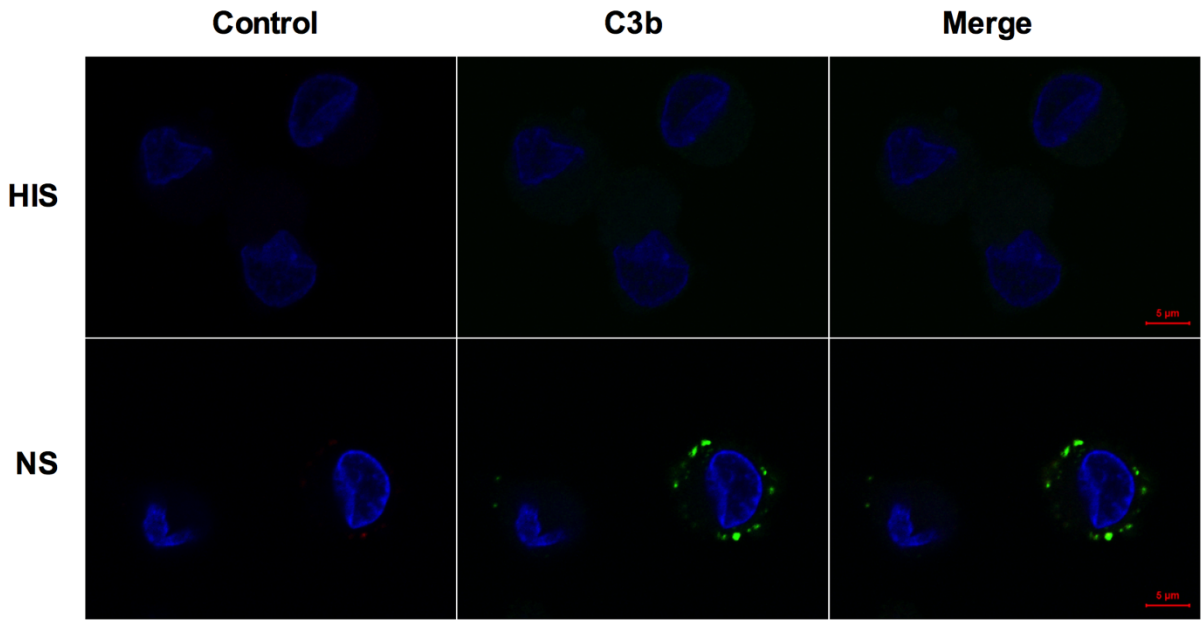
Supplementary Figures

Supplementary Figure 1



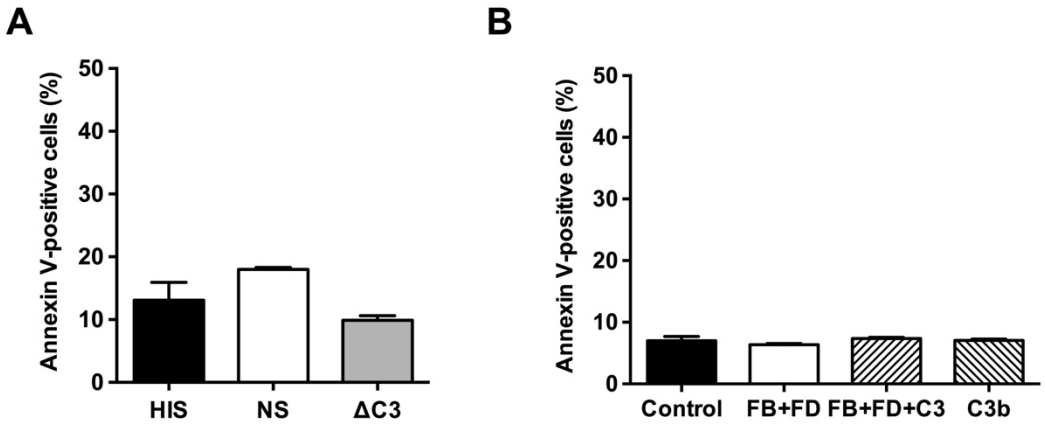
Supplementary Figure S1. Expression of endocytic receptors on MO-DCs incubated in normal serum. Five-day-old immature MO-DCs (2.10^5 cells in RPMI-1640) were incubated in 20% normal (NS) or heat-inactivated serum (HIS) for 1 hr at 37°C. The expression of LRP (CD91), MMR (CD206) and DC-SIGN (CD209) was analyzed by flow cytometry. Results are means±SD of 4 independent experiments. Statistical significance was assessed using the double-sided nonparametric Mann-Whitney test (ns: not significant).

Supplementary Figure 2



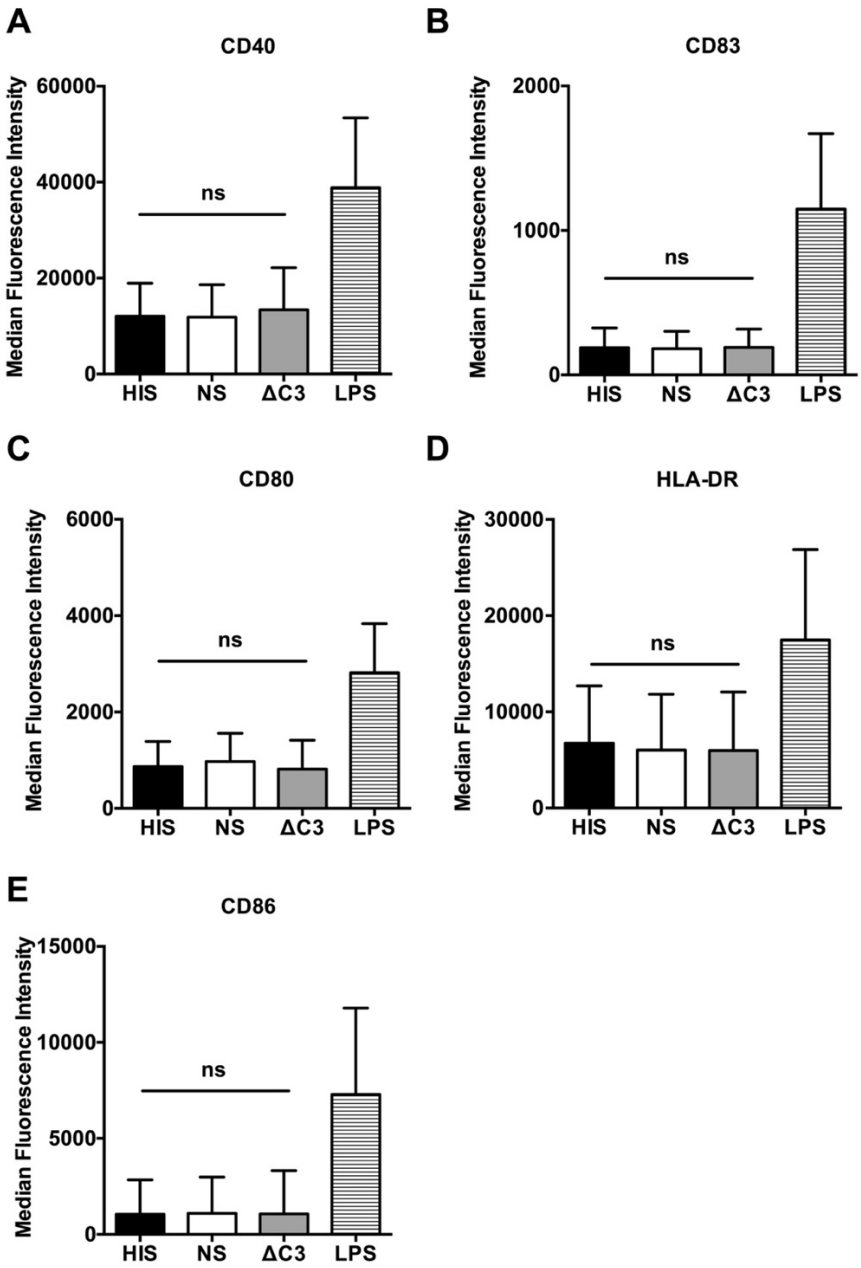
Supplementary Figure S2. Co-localization of FVIII and C3b on MO-DCs. In control experiments, normal (NS) or heat-inactivated serum (HIS) were incubated in the absence of FVIII for 1 hr at 37°C and then with MO-DCs for 2 hr at 37°C. C3b was detected with polyclonal anti-C3b/iC3b. Cells were also incubated with anti-human FVIII antibodies. Cells were then incubated with appropriate fluorescently labelled secondary antibodies (FVIII: red; C3b: green). Deposition of C3b at the cell surface at the surface of MO-DC was analysed by confocal microscopy.

Supplementary Figure 3



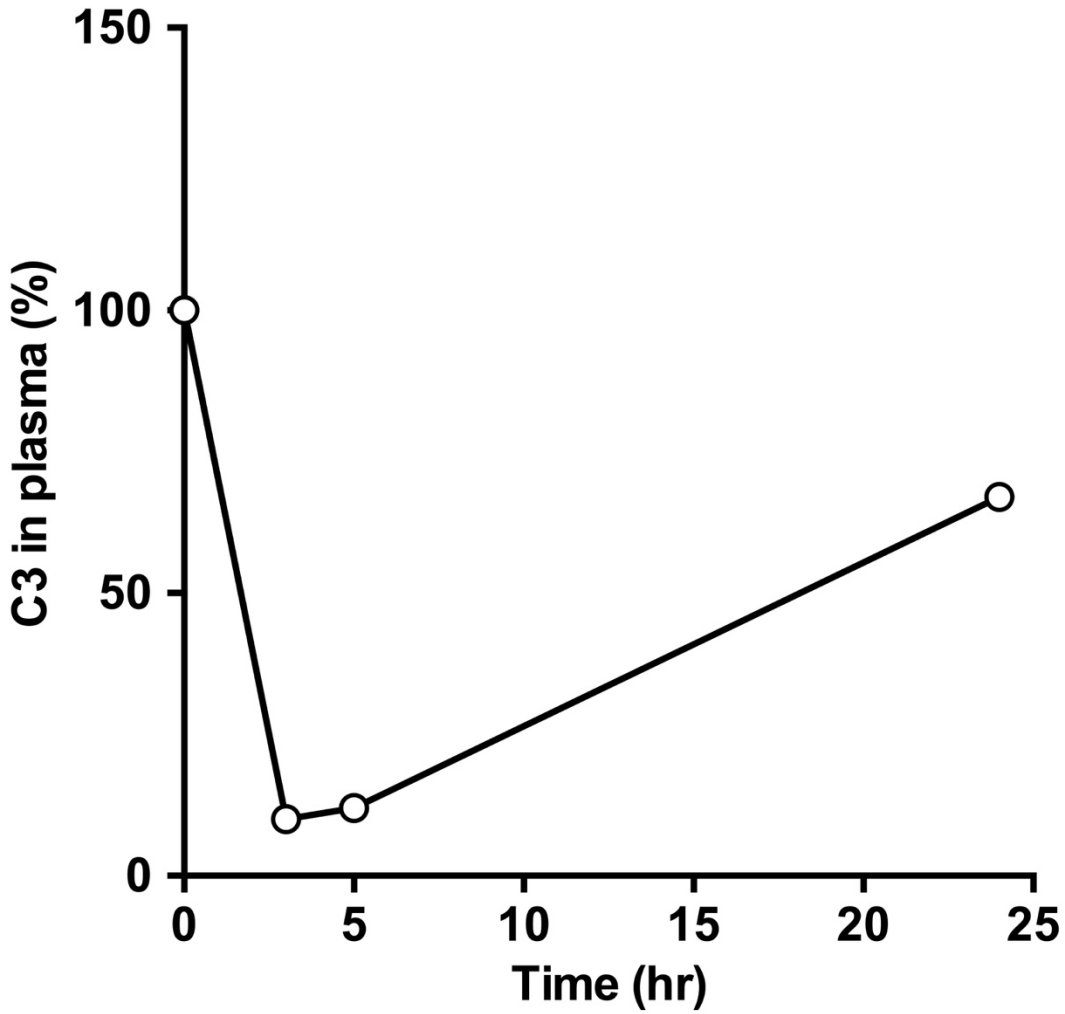
Supplementary Figure S3. Lack of apoptosis of MO-DCs incubated with the C3 convertase. Panel A. Five day-old immature MO-DCs (2.10^5 cells in RPMI-1640) were incubated in 20% normal serum (NS), heat-inactivated serum (HIS) or C3-depleted serum (ΔC3) for 2 hr at 37°C. Panel B. MO-DCs (2.10^5 cells in X-VIVO[15]) were incubated alone (Control), in the presence of factor B (FB, 50 µg/ml) and factor D (FD, 1 µg/ml) with or without C3 (250 µg/ml), or in the presence of C3b (250 µg/ml) for 2 hr at 37°C. Cells were stained for phosphatidylserine surface expression using APC-labeled annexin-V.

Supplementary Figure 4



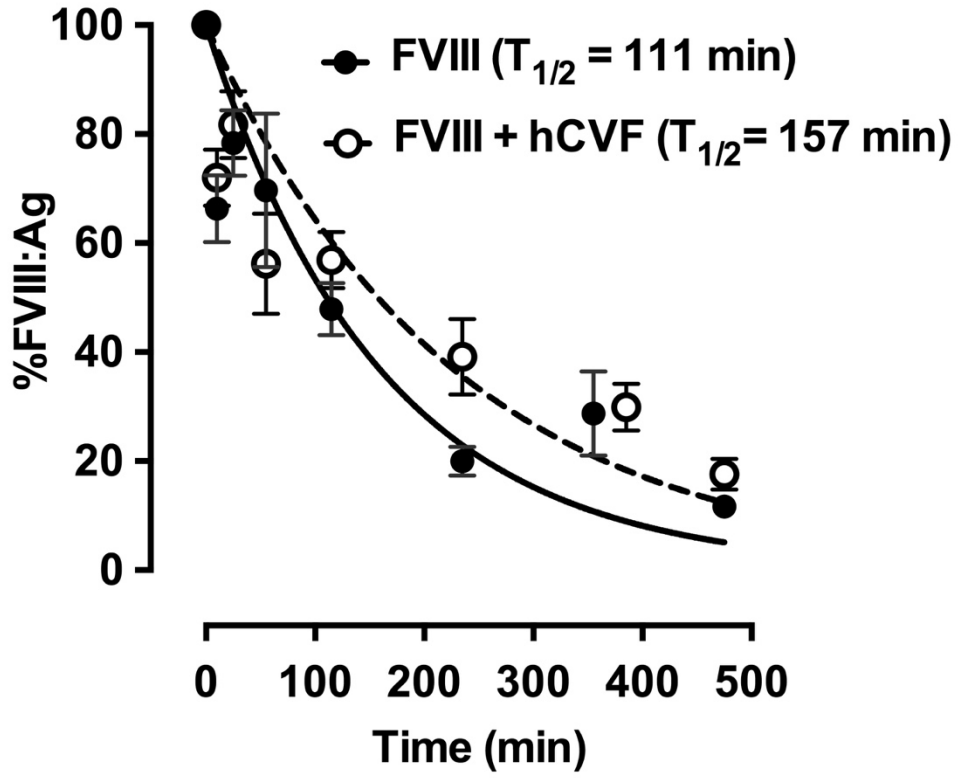
Supplementary Figure S4. Complement does not induce the maturation of dendritic cells in the presence of FVIII *in vitro*. Five-day-old immature MO-DCs (2.10^5 cells in RPMI-1640) were cultured with FVIII (35 nM) and 10% normal serum (NS), heat-inactivated serum (HIS), C3-depleted serum (ΔC3), or with LPS (1 µg/ml) at 37°C for 24 hr. The expression of different maturation markers was assessed as MFI by flow cytometry. Dead cells were excluded with propidium iodide staining (5 µg/ml). Graphs depict the MFI measured for CD40 (panel A), CD83 (panel B), CD80 (panel C), HLA-DR (panel D) and CD86 (panel E). Means±SD are from 7 independent experiments. Statistical significance was assessed using the double-sided nonparametric Mann-Whitney test (ns: non-significant).

Supplementary Figure S5

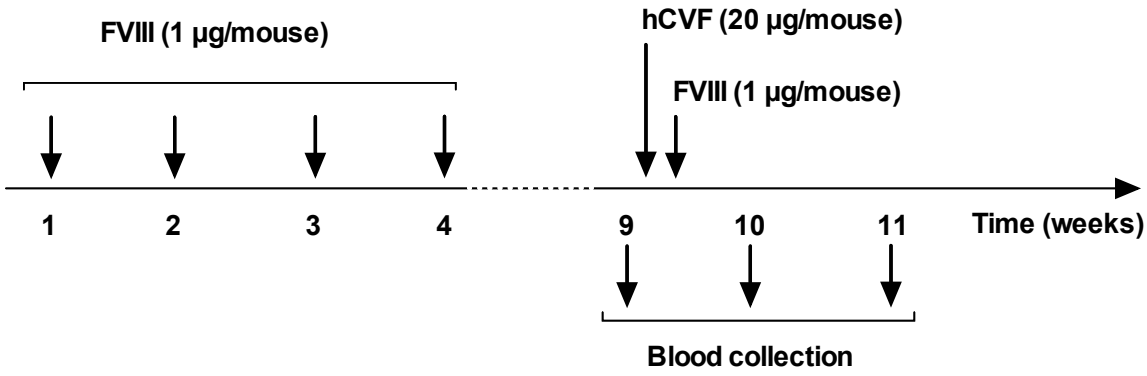


Supplementary Figure S5. Efficacy of complement depletion by hCVF in FVIII-deficient mice. The time-course of C3 depletion by hCVF (20 μg) after infusion into FVIII-deficient mice was measured in the plasma 3, 5 and 24 hr after hCVF injection using an ELISA.

Supplementary Figure 6



Supplementary Figure S6. Half-life of FVIII in FVIII-deficient mice upon complement depletion. FVIII-deficient mice were treated with PBS (full circles) or hCVF (20 µg, empty circles) to deplete complement. Six hours later, mice were administered with FVIII (10 nM). Residual FVIII antigen (FVIII:Ag) was measured using ELISA at different time points following FVIII administration (n=4-6 mice per time point). Data are plotted as a percentage of the initial FVIII level (measured 5 minutes after administration) versus time and represent means±SEM. Experimental data were fitted with the one-phase exponential decay equation ($R^2 \geq 0.63$).



Supplementary Figure S7. Effect of hCVF in the recall anti-FVIII immune response in FVIII-primed FVIII-deficient mice. FVIII-deficient mice were injected once a week for 4 weeks with FVIII (1 µg/mouse). At week 9, mice were treated with either 20 µg hCVF or PBS, and 6 hours later with FVIII. Blood was collected before hCVF/PBS injection and at week 10 and 11.