Supplemental material

Title: Modification of an exposed loop in the C1 domain reduces immune responses to factor VIII in hemophilia A mice

Authors: Aleksandra Wroblewska, Simon D. van Haren, Eszter Herczenik, Paul Kaijen, Aleksandra Ruminska, Sheng-Yu Jin, X. Long Zheng, Maartje van den Biggelaar, Anja ten Brinke, Alexander B. Meijer, Jan Voorberg

Supplemental methods

Blocking experiments

To block the uptake of FVIII by human monocyte-derived macrophages (MDMΦ), prior incubation with cells (30 minutes, 37°C), 40 nM of monoclonal antibody VK34 or KM33 was first incubated with 10 nM FVIII for 30 minutes at 37°C.

Binding of FVIII to recombinant human and mouse VWF

Recombinant human VWF was purified as described before.[1] Recombinant mouse VWF was prepared as reported previously.[2] FVIII binding to VWF was measured by ELISA. Briefly, Nunc-Maxisorp 96-well plates were coated with 5 µg/ml either recombinant human or mouse VWF in 50 mM $NaHCO_3$ (pH 9.8) overnight at 4°C. Subsequently, FVIII variants were incubated on the plate in binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$ 2% HSA) in a concentration range up to 20 U/ml. Bound FVIII was detected with HRP-conjugated mAb CLB-CAg12 (targeting the A3-C1 domains of FVIII). Optical densities were measured at 450 nm with the subtraction of values obtained at 540 nm.

Surface plasmon resonance analysis

Surface plasmon resonance analysis was performed using a BIAcore3000 biosensor system (GE Healthcare, Uppsala, Sweden). For interaction studies of FVIII with VWF, mouse VWF was covalently coupled to the dextran surface of an activated CM5-sensor chip. Subsequently, various concentrations of FVIII variants (0.15-2 nM) were passed over immobilized VWF (10 fmol/mm^2) at a flow rate of 20 µl/min. The interaction between FVIII and LRP was studied by using recombinant LRP1 cluster II. [3] First, FVIII was immobilized onto a CM5 sensor chip precoated with FVIII C2 domain–targeting mAb EL14 (27 fmol/mm^2). Then, various concentrations of LRP1 cluster II (6.25-200 nM) were passed over the immobilized FVIII with a flow rate of 20 µL/min. For both FVIII-VWF as

well as FVIII-LRP interaction, nonlinear regression was used to fit the obtained data to a one-phase exponential association equation with GraphPad Prism 5.0 software (San Diego, Calif). The responses at equilibrium (Ymax) of each concentration of either FVIII (FVIII-VWF interaction) or LRP1 cluster II (FVIII-LRP binding) were then fitted by nonlinear regression using a one-site binding hyperbola to calculate apparent K_D values. For binding of FVIII to various monoclonal antibodies, FVIII was immobilized onto CM5 sensor chip precoated with mAb EL14 (27 fmol/mm^2). Association and dissociation of 100 nM full-length antibody KM33 (targeting C1 domain of FVIII), ESH4 (C2 domain), CLB-CAg12 (A3C1 domain) or CLB-CAg9 (A2 domain) were performed in the same buffer at a flow rate of 20 µl/min for 4 minutes at 25°C. Association and dissociation curves were corrected for nonspecific binding to a channel coated with EL-14 only.

CD4$^+$ T cell proliferation assay

Spleens collected from E17KO mice after 5 times weekly i.v. injections of 1 µg FVIII were processed into single-cell suspensions. Erythrocytes were removed and CD8$^+$ cells were depleted by magnetic bead separation using beads coated with anti-mouse CD8 antibody. Remaining CD8$^-$ cells were cultured in round-bottomed 96-well plates for 48 hours in X-VIVO 15 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 55 µM 2-mercaptoethanol in presence of FVIII (0.5 µg/ml), FVIII peptides (10 µg/ml) or concanavalin A (1 µg/ml). Proliferation was assayed by the addition of 1 µCi/well of [^{3}H]thymidine for the last 18-20 hours. The results are expressed as counts per minute (cpm) of cells incubated with FVIII or peptides. FVIII peptides used in the assay: 2191-2210 (FVIII C2 domain), 2201-2220 (C2), 2211-2230 (C2), 2201-2230 (C2), 2076-2105 (C1), 2081-2100 (C1). FVIII C1 peptides contained either wild-type (WT) or mutated sequence (AAA: residues 2090, 2092 and 2093 were replaced by an Ala).

Supplemental results

Endocytosis of FVIII by human monocyte-derived macrophages is mediated by the C1 domain.

Monoclonal antibody KM33 blocked the uptake of FVIII by MDMΦ whereas monoclonal antibody VK34 did not affect uptake of FVIII by MDMΦ (Figure S1). These results show that uptake of FVIII by MDMΦ is also mediated via its C1 domain.

Binding kinetics of FVIII variants to human and murine VWF and LRP

To study the binding kinetics between murine VWF, LRP and FVIII, we performed surface plasmon resonance experiments, where either various concentrations of receptor fragment were passed over the sensor chip immobilized with FVIII or various FVIII concentrations were passed over immobilized VWF. Both FVIII WT and well as FVIII-R2090A/K2092A/F2093A were binding murine and human VWF in dose-dependent manner (Figure S2, A and B). Binding of FVIII WT and FVIII-R2090A/K2092A/F2093A to murine VWF was studied in more detail using surface plasmon resonance analysis. Different concentration of FVIII WT and FVIII-R2090A/K2092A/F2093A were passed over immobilized murine VWF (Figure S2, C and D, respectively). A dose dependent increase in binding of both FVIII WT and FVIII-R2090A/K2092A/F2093A was observed. The responses at equilibrium were used to calculate the binding parameters. Maximal binding was slightly lower for FVIII-R2090A/K2092A/F2093A when compared to FVIII WT. The apparent dissociation constant were below 1 nM for both WT FVIII and FVIII-R2090A/K2092A/F2093A (Figure S2, E and F), indicating that the binding affinity of FVIII-R2090A/K2092A/F2093A to mouse VWF is similar to that of WT FVIII.

We also determined the affinity of FVIII-R2090A/K2092A/F2093A for cluster II of LRP. We observed reduced binding for FVIII-R2090A/K2092A/F2093A to LRP1 cluster II when compared to WT FVIII (Figure S3). Calculated apparent K_D value was significantly higher for FVIII-R2090A/K2092A/F2093A when compared to that of WT FVIII (Figure S3, D).

Binding of a panel of antibodies to FVIII, FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A

To exclude the possibility that observed differences in binding to KM33 is determined only by different binding of FVIII to EL-14, a panel of anti-FVIII monoclonal antibodies (KM33 – C1 domain, ESH4 – C2 domain, CLB-CAg12 – A3C1 domain, CLB-CAg9 – A2 domain) was passed over FVIII WT, FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A immobilized on sensor chip via C2-targeting antibody EL-14. Only binding to KM33 was altered for FVIII mutants, while other antibodies showed similar binding to all three FVIII variants tested (Figure S4). These data show that FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A react in a similar manner with EL-14, ESH4, CLB-CAg12 and CLB-CAg 9. These findings also suggest that no major structural changes have occurred as a result of the substitution of residues 2090, 2092 and 2093 by an Ala.

Modification of residues Arg2090, Lys2092 and Phe2093 does not alter a major $CD4^+$ T cell epitope

Reduced immune response observed for FVIII-R2090A/K2092A/F2093A in hemophilia mice could potentially result from elimination of a dominant T cell epitope. Therefore, we verified whether $CD4^+$ T cells directed towards an epitope including these residues were present in hemophilia mice following multiple infusions with FVIII. CD8-depleted

splenocytes isolated from immunized E17KO mice were stimulated either with FVIII C1 domain peptides 2076-2115 and 2081-2100 corresponding to either wild-type FVIII or FVIII-R2090A/K2092A/F2093A. In these latter peptides residues 2090, 2092 and 2093 were replaced by Ala. As a control we used peptides covering an established immunodominant T cell epitope in the C2 domain (region 2191-2230).[4] FVIII and ConA were used as controls. In agreement with previous studies, proliferation in the presence of FVIII peptides was modest. Nevertheless, significant proliferation was observed only for C2 peptides (Figure S5). No specific proliferation was observed upon incubation of CD8-depleted splenocytes with peptides 2076-2105 and 2081-2100 (Figure S5). Also peptides 2076-2105 and 2081-2100 in which residues 2090, 2092 and 2093 were replaced by an Ala did not induce $CD4^+$ T cell proliferation (Figure S5). These data suggest that region encompassing residues Arg2090, Lys2092 and Phe2093 does not comprise major $CD4^+$ T cell epitope. Our findings indicate that the reduced immune response observed for FVIII-R2090A/K2092A/F2093A in hemophilia A mice is not caused by elimination of a dominant T cell epitope.

Supplemental references

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2. Xiao J, Jin SY, Xue J, Sorvillo N, Voorberg J, Zheng XL. Essential domains of a disintegrin and metalloprotease with thrombospondin type 1 repeats-13 metalloprotease required for modulation of arterial thrombosis. *Arterioscler Thromb Vasc Biol.* 2011;31(10):2261-9.

3. Bovenschen N, Boertjes RC, van Stempvoort G, et al. Low density lipoprotein receptor-related protein and factor IXa share structural requirements for binding to the A3 domain of coagulation factor VIII. *J Biol Chem.* 2003;278:9370-7.

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Supplemental figure legends

Figure S1. Endocytosis of FVIII by MDMΦ is mediated by C1 domain. FVIII WT (10 nM) was preincubated either with C1-targeting antibody KM33 (grey bar) or A2-targeting VK34 (black bar) and subsequently added to MDMΦ. Internalized FVIII was detected using CLB-CAg117-FITC antibody in the presence of saponin. Results are expressed as % MFI, where 100% corresponds to mean fluorescence intensity obtained with FVIII alone (white bar). Mean ± SD are shown of three independent experiments. Representative histograms (grey filled histograms show untreated cells, open histograms represent cells treated with FVIII in presence or absence of antibodies) are shown on the right.

Figure S2. Binding of FVIII to recombinant VWF. (A and B) FVIII WT (●, solid line) and FVIII-R2090A/K2092A/F2093A (○, dotted line) were incubated with human (A) or murine (B) VWF-coated plates. Bound FVIII was detected with A3-C1 domain-targeting antibody CLB-CAg12. Mean ± SD are shown of three independent experiments and expressed as % maximum binding. WT – FVIII wild-type; 2090/92/93 – FVIII-R2090A/K2092A/F2093A. (C and D) Various concentrations (0.15-2 nM) of FVIII WT (C) or FVIII-R2090A/K2092A/F2093A (D) were passed over immobilized murine VWF. Dissociation was initiated upon replacement of ligand solution by buffer. The responses at equilibrium (Ymax) of each concentration of either FVIII WT or FVIII-R2090A/K2092A/F2093A (E) were used to calculate the apparent K_D and B_{MAX} values (F). Experiments were performed in duplicate.

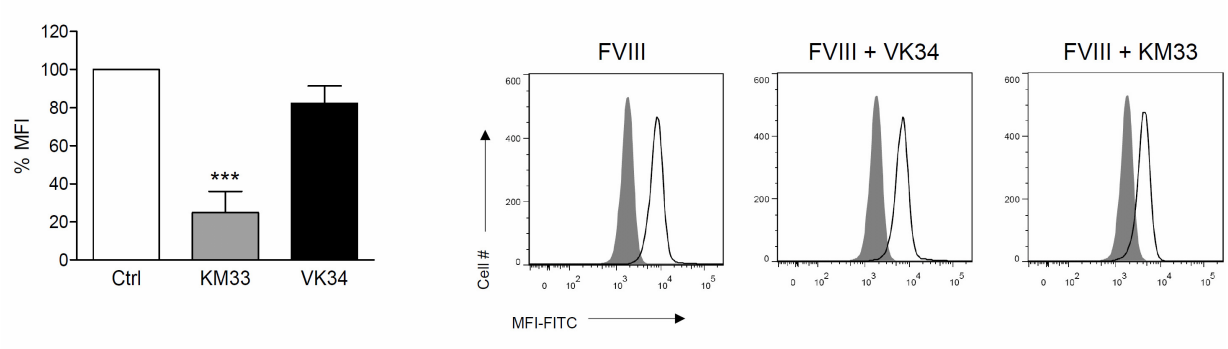
Figure S3. Binding of FVIII to LRP1 cluster II. (A and B) Various concentrations (6.25, 12.5, 25, 50, 100 and 200 nM) of LRP1 cluster II were passed over immobilized FVIII

WT (A) or FVIII-R2090A/K2092A/F2093A (B). Dissociation was initiated upon replacement of ligand solution by buffer. Ymax values obtained from one-phase exponential association alignments of LRP1 cluster II (C) binding curves were used to calculate the apparent K_D and B_{MAX} values (D). Experiments were performed in triplicate.

Figure S4. Surface plasmon resonance analysis of FVIII interaction with various anti-FVIII monoclonal antibodies. Antibodies KM33, ESH4, CLB-CAg12 or CLB-CAg9 (100 nM) were passed over FVIII WT (solid line), FVIII-K2092A/F2093A (dashed line) or FVIII-R2090A/K2092A/F2093A (dotted line) immobilized on a sensor chip via C2 domain-targeting monoclonal antibody EL-14. Dissociation was initiated upon replacement of ligand solution by buffer. Data are representative of 3 independent experiments.

Figure S5. $CD4^+$ T cells from immunized E17KO mice recognize FVIII C2 domain-derived peptides, but respond poorly to C1 peptides. $CD8^-$ splenocytes of hemophilia A mice injected with FVIII were assayed in a thymidine (^3H) incorporation assay. Proliferation was measured after 48 hours and thymidine was added for the last 18-20 hours. Results are shown as counts per minute (cpm) from triplicate wells (mean ± SD). Ctrl indicates unstimulated cells. Black bars denote proliferation in response to peptides containing an established T cell epitope in the C2 domain. Grey bars represent proliferation in response to peptides overlapping residues Arg2090, Lys2092 and Phe2093. Proliferation in response to FVIII and ConA was included as a control.

Figure S1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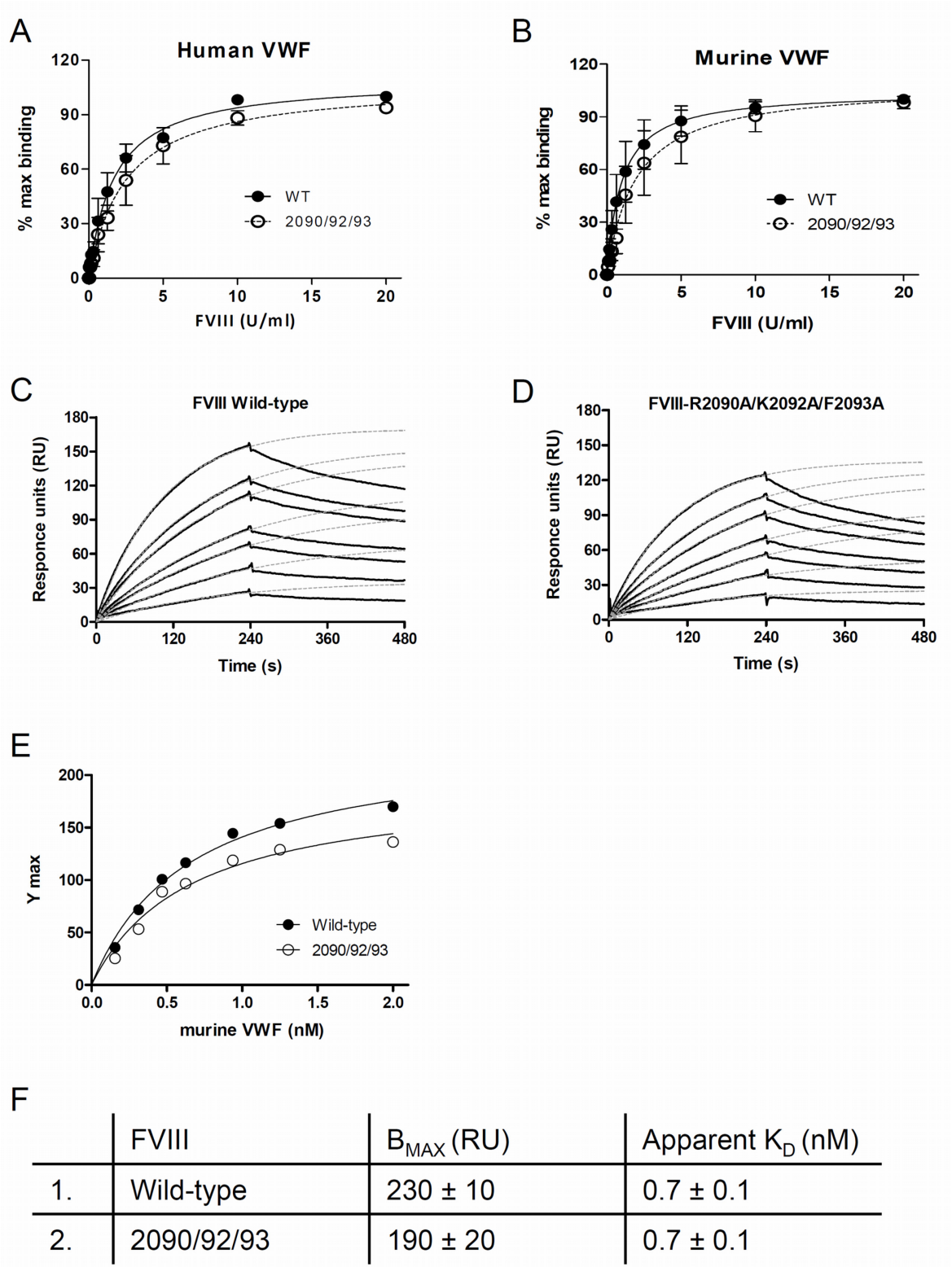
FVIII + KM33
Cell #
MFI-FITC

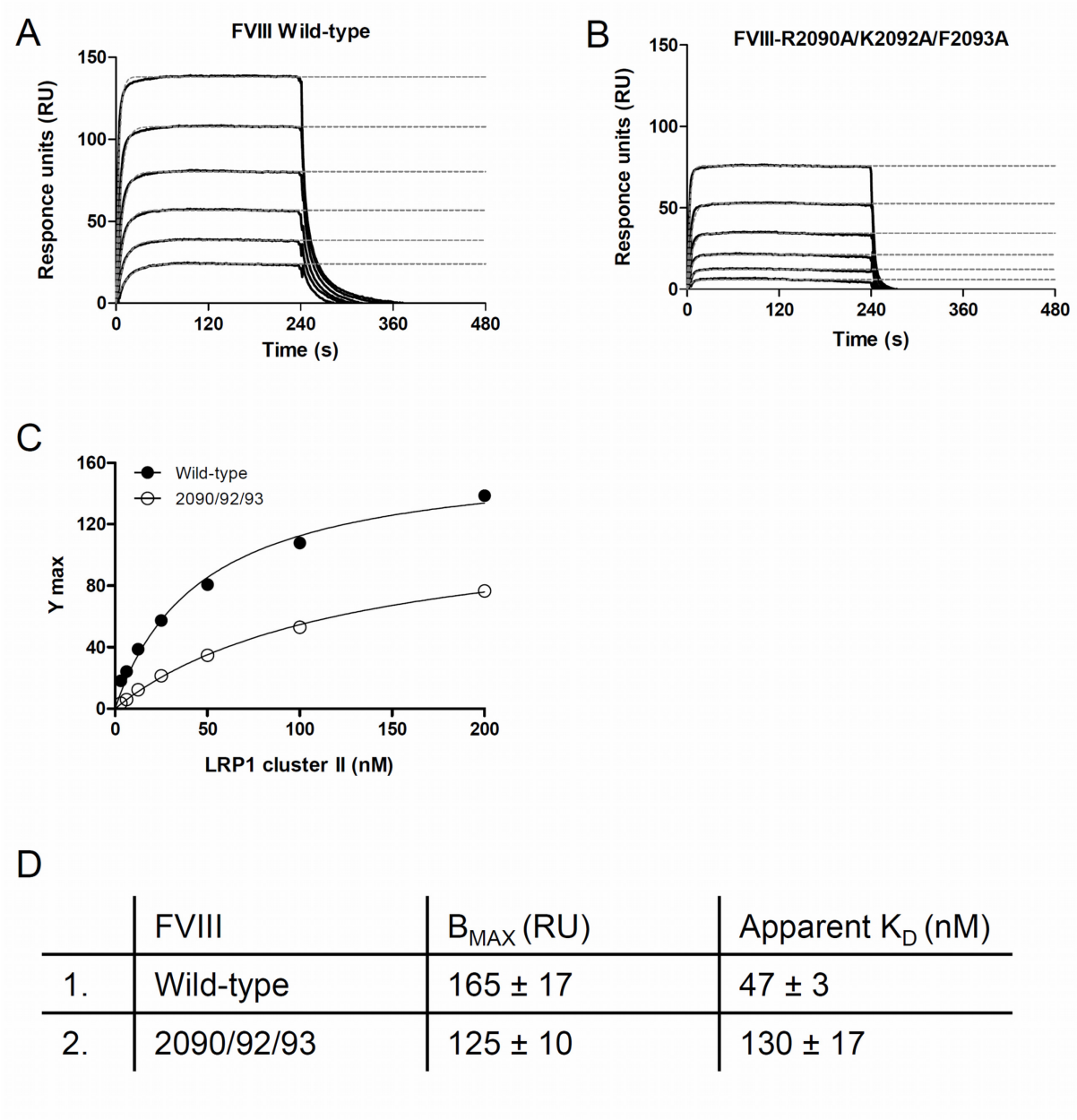
Figure S2



F

	FVIII	B_{MAX} (RU)	Apparent K_D (nM)
1.	Wild-type	230 ± 10	0.7 ± 0.1
2.	2090/92/93	190 ± 20	0.7 ± 0.1

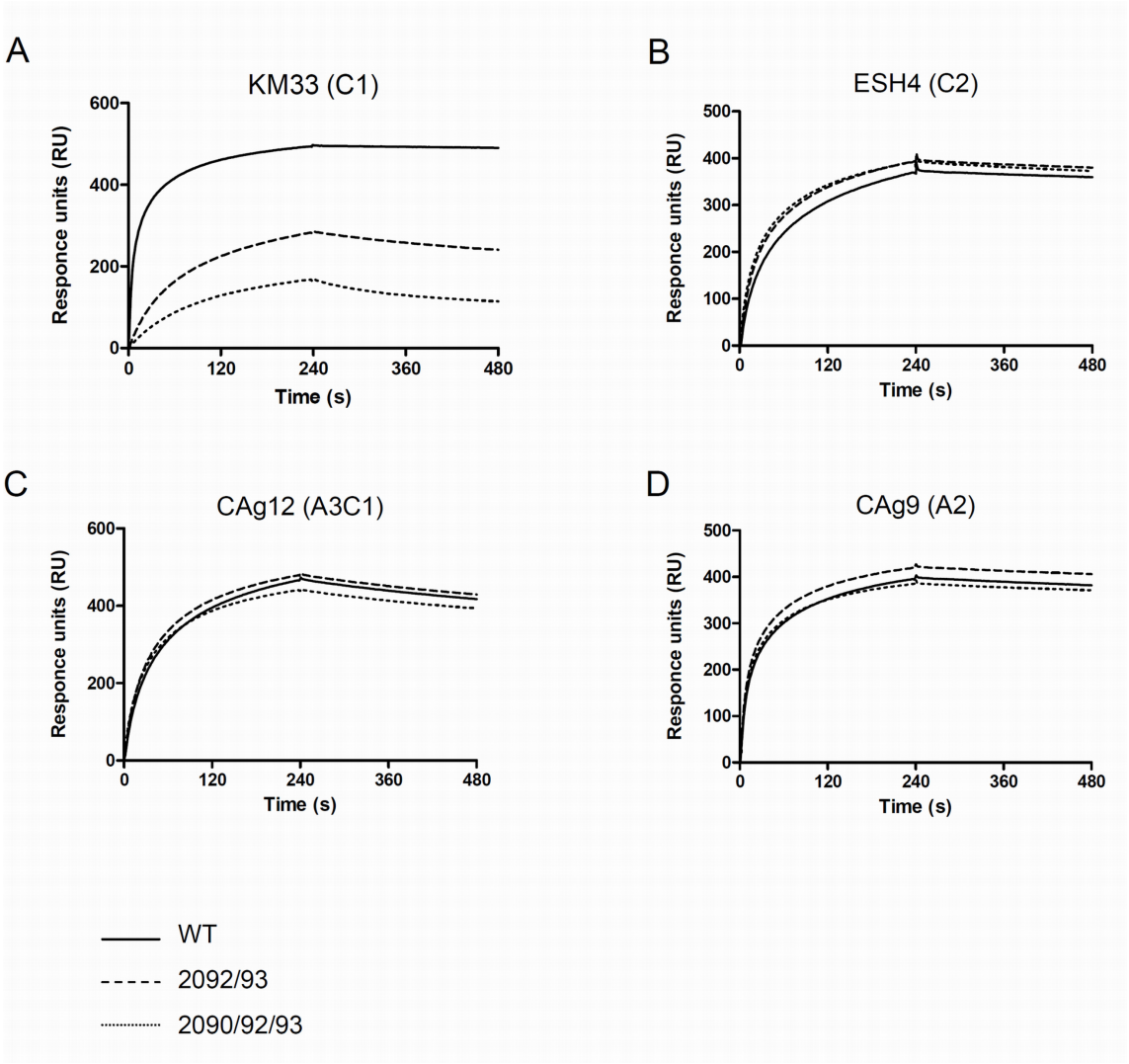
Figure S3



D

	FVIII	B_{MAX} (RU)	Apparent K_D (nM)
1.	Wild-type	165 ± 17	47 ± 3
2.	2090/92/93	125 ± 10	130 ± 17

Figure S4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Time (s)
B
ESH4 (C2)
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Time (s)
C
CAg12 (A3C1)
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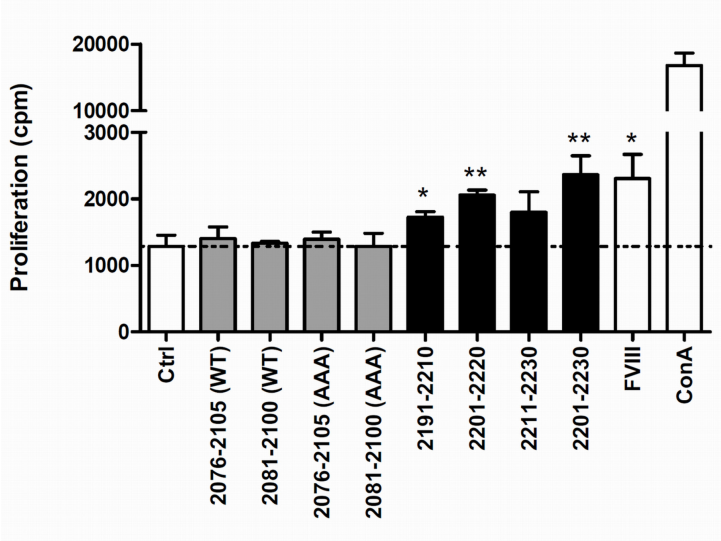
Time (s)
WT
2092/93
2090/92/93

Figure S5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2076-2105 (AAA)
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2191-2210
2201-2220
2211-2230
2201-2230
FVIII
ConA