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

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ABSTRACT

The development of inhibitory antibodies to therapeutic factor VIII is the major complication of replacement therapy in patients with hemophilia A. The first step in the initiation of the anti-factor VIII immune response is factor VIII interaction with receptor(s) on antigen-presenting cells, followed by endocytosis and presentation to naïve CD4+ T cells. Recent studies indicate a role for the C1 domain in factor VIII uptake. We investigated whether charged residues in the C2 domain participate in immunogenic factor VIII uptake. Co-incubation of factor VIII with BO2C11, a monoclonal C2-specific immunoglobulin G, reduced factor VIII endocytosis by dendritic cells and presentation to CD4+ T cells, and diminished factor VIII immunogenicity in factor VIII-deficient mice. The mutation of basic residues within the BO2C11 epitope of C2 replicated reduced *in vitro* immunogenic uptake, but failed to prevent factor VIII immunogenicity in mice. BO2C11 prevents factor VIII binding to von Willebrand factor, thus potentially biasing factor VIII immunogenicity by perturbing its half-life. Interestingly, a factor VIII^{Y1680C} mutant, that does not bind von Willebrand factor, demonstrated unaltered endocytosis by dendritic cells as well as immunogenicity in factor VIII-deficient mice. Co-incubation of factor VIII^{Y1680C} with BO2C11, however, resulted in decreased factor VIII immunogenicity *in vivo*. In addition, a previously described triple C1 mutant showed decreased uptake *in vitro*, and reduced immunogenicity *in vivo*, but only in the absence of endogenous von Willebrand factor. Taken together, the results indicate that residues in the C1 and/or C2 domains of factor VIII are implicated in immunogenic factor VIII uptake, at least *in vitro*. Conversely, *in vivo*, the binding to endogenous von Willebrand factor masks the reducing effect of mutations in the C domains on factor VIII immunogenicity.

Introduction

Hemophilia A is a monogenic disorder associated with mutations causing reductions in functional levels of coagulation factor VIII (FVIII). FVIII consists of a heavy chain (A1-a1-A2-a2-B domain) and a light chain (a3-A3-C1-C2) held together by non-covalent interactions.[1] It rapidly associates with von Willebrand factor (VWF) in blood, and this interaction is necessary for maintaining its circulatory half-life.[2] Current treatment for FVIII deficiency requires prophylactic infusions of plasma-derived or recombinant FVIII. However, up to 30% of patients with severe hemophilia A develop an anti-FVIII immune response, thus rendering treatment ineffective.[3] The development of anti-FVIII antibody responses is dependent on T helper cells, requiring antigen uptake and presentation by antigen-presenting cells (APCs).[4]

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Hence, understanding the initial steps involved in FVIII capture by APCs may provide novel strategies to prevent the onset of the immune response.

Several groups, including ours, have investigated the endocytic pathways involved in FVIII uptake. Candidate receptors such as macrophage mannose receptor (MMR, CD206), low-density lipoprotein receptor-related protein (LRP, CD91), or other receptor-associated protein (RAP)-sensitive receptors have been proposed.[5-10] Equally, the nature of the residues within FVIII domain(s) that contribute to these interactions is also an area of active investigation. Despite these efforts, the *in vivo* relevance of these receptors and the nature of the FVIII residues involved in FVIII uptake remain unclear. Recently, Herczenik *et al.*[10] demonstrated that KM33, a human C1 domain-specific monoclonal immunoglobulin G (IgG), inhibits FVIII endocytosis by monocyte-derived dendritic cells (MoDCs) or mouse bone marrow-derived dendritic cells (BMDCs). KM33 engages K2092, F2093 and R2090 residues, involved in the interactions with phospholipid membrane surfaces.[11] Additionally, KM33 inhibits interactions of the C1 domain with membrane surfaces, VWF and LRP.[12] FVIII uptake by LRP in MoDCs, used as model APCs, has been ruled out,[8] while a role for CD206 has been controversially documented.[7,9,10] This suggests that FVIII uptake by APCs may involve other endocytic receptor(s). Importantly, FVIII mutants containing alanine substitutions of the K2092, F2093 and R2090 C1 residues, exhibit diminished uptake *in vitro* and reduced immunogenicity in a mouse model of severe hemophilia A.[11] Together, these results point to the significance of membrane-binding residues within the C1 domain for FVIII uptake both *in vitro* and *in vivo*. Similar to the C1 domain, the C2 domain of FVIII interacts with phospholipid membrane surface, a binding that involves several basic residues.[13]

In the study herein, we investigated whether membrane-interacting residues within the C2 domain of FVIII are involved in FVIII uptake. We first show that BO2C11, a well-characterized human monoclonal IgG that engages membrane-binding residues in the C2 domain,[14] inhibits FVIII uptake and presentation *in vitro*, and reduces FVIII immunogenicity *in vivo*. We also demonstrate that this reduced immunogenicity is independent of the ability of FVIII to interact with endogenous VWF. Additionally, by site-directed mutagenesis, we demonstrate that the R2215 residue, which is part of the BO2C11 epitope, is implicated in the cellular uptake of FVIII by APCs *in vitro*. Together with the published data, our results suggest a potential synergy between membrane-binding residues in both the C1 and C2 domains of FVIII in mediating FVIII recognition or uptake by APCs *in vitro*. Furthermore, we also demonstrate that FVIII C domain mutations exhibit diminished immunogenicity *in vivo* only in the absence of endogenous VWF.

Methods

Reagents

Recombinant human FVIII (Refacto) came from Pfizer (New York, NY, USA). Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) came from Cellgenix Technology Transfer (Freiburg, Germany). The monoclonal mouse anti-FVIII (mAb6), the human anti-A2 (BO2BII) and anti-C2 (BO2C11) domains antibodies were kind gifts from Drs JM Saint-Remy and M Jacquemin (KUL, Leuven, Belgium). The monoclonal human-derived anti-C1 (KM33) was a gift from Dr J Voorberg (Sanquin, Amsterdam, The Netherlands). The mouse monoclonal anti-A2 domain (GMA-8015) and anti-C2 domain (ESH8) antibodies were purchased from Green Mountain Antibodies (Burlington, VT, USA) and Sekisui Diagnostics (Kings Hill, Kent, UK), respectively. Biotin-labeled GMA-8015 was prepared upon incubation with a 20-fold molar excess of EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Courtaboeuf, France) for 30 min at room temperature, and the removal of excess biotin by diafiltration was carried out using 30 kDa Amicon Ultra-15 centrifugal filter units (Merck Millipore, Saint-Quentin-en-Yvelines, France). BO2C11 fragment antigen binding (Fab) or $F(ab')_2$ fragments were digested by papain or pepsin following the manufacturer's instructions (Thermo Fisher Scientific, Courtaboeuf, France).

Production and purification of recombinant mutated or wild-type FVIII

Complementary DNA (cDNA) encoding human B-domain deleted (BDD) FVIII ($FVIII^{HSQ}$), containing the 14-amino acid segment SFSQNPPVLKRHQR in place of the B domain, cloned in the ReNeo mammalian expression plasmid with a geneticin resistance, has been described previously.[15] The cDNA encoding $FVIII^{HSQ}$ was used as a template to generate the R2215A, R2220A, R2215A-R2220A, R2090A-K2092A-F2093A and Y1680C FVIII mutants by splicing by overlap extension mutagenesis as described in the *Online Supplementary Methods*. The 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 in the *Online Supplementary Methods*. The concentration of purified wild-type and mutated FVIII 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. Specific activities were estimated by a one-stage clotting assay and ranged between 4800-9000 IU/mg. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) migration profiles of the different purified recombinant FVIII are shown in the *Online Supplementary Figure S1*. In parallel, the ability of the different FVIII molecules to generate activated factor X was assessed in a chromogenic assay (Siemens Healthcare Diagnostics, Marburg, Germany).

FVIII binding to VWF and monoclonal antibodies

Ninety-six-well ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with human plasma-derived VWF (Wilfactin, LFB, Les Ulis, France), BO2C11, BO2BII, ESH8 or KM33 at 1 µg/ml in bicarbonate buffer, pH 9.5, for 1 hr at 37°C. Wells were blocked with 20 mM HEPES, 0.15 M NaCl, 0.05% Tween 20 and 5% bovine serum albumin (BSA), pH 7.4. Wild-type and mutated FVIII were then diluted in blocking buffer and incubated in the coated wells for 1 hr at 37°C. Bound FVIII was revealed using either biotinylated GMA-8015 (1 µg/ml), followed by streptavidin conjugated to horseradish peroxidase (R&D systems, Lille, France) or, in the case of BO2BII-bound FVIII, with ESH8 followed by a horseradish peroxidase conjugated polyclonal goat anti-mouse IgG antibody (Southern Biotech, Anaheim, CA, USA) and the o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, Saint-Louis, MO, USA). Absorbance was read at 492 nm.

Generation of immature human MoDCs and mouse BMDCs

Monocytes were isolated from the blood of healthy donors using anti-$CD14^+$ magnetic microbeads (Miltenyi Biotec, Paris,

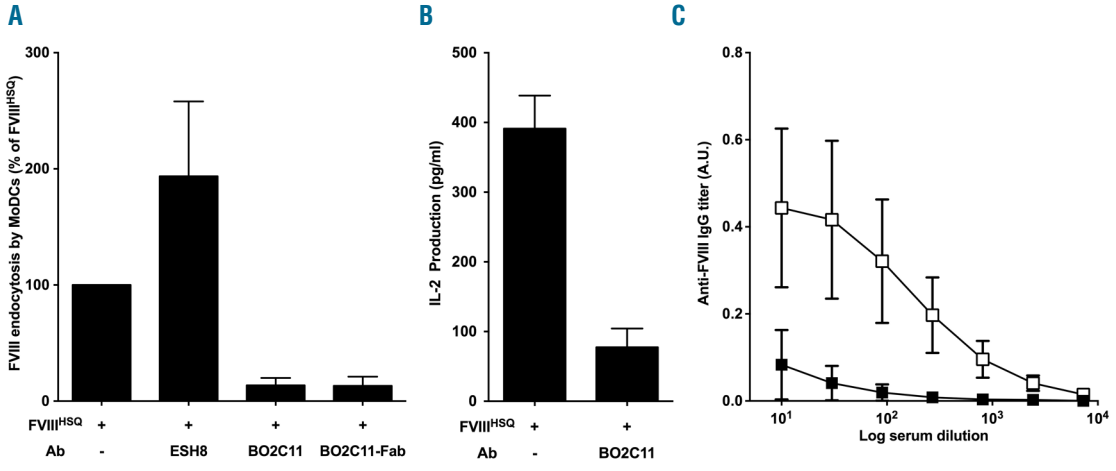

Figure 1. The anti-C2 antibody BO2C11 inhibits FVIII uptake *in vitro* and modulates FVIII immunogenicity *in vivo*. Panel A. B domain-deleted FVIII (20 nM) was pre-incubated alone or with equimolar concentrations of ESH8, BO2C11 or with a 2 molar excess BO2C11 Fab fragments. Uptake by human MoDCs was 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 ($FVIII^{HSQ}$) incubated alone. The graph is representative of 4 independent donors (mean±SEM). Panel B. Immature MoDCs 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 alone or pre-incubated with 2 molar excess of BO2C11. Activation of T cells was measured by IL-2 secretion in the supernatant by ELISA (BD Biosciences). The graph is representative of 4 experiments (mean±SEM). Panel C. Hemophilic FVIII exon 16 knock-out mice (n=6/group) were injected once a week for 4 weeks with 0.2 µg of B domain-deleted FVIII pre-incubated with 6 µM $F(ab')_2$ of BO2C11 (closed square) or a human IgG4 isotype control (open square). After 4 weeks, the anti-FVIII antibody response was measured. Anti-FVIII IgG titers are defined as arbitrary units using the mouse monoclonal anti-FVIII IgG mAb6 as a standard. Data are represented as serum dilution *versus* mean±SEM of absorbance (492 nm). FVIII: factor VIII; MoDCs: monocyte-derived dendritic cells; A.U.: arbitrary unit; IL-2: interleukin-2; IgG: immunoglobulin G; Ab: antibody.

France). Ethics committee approval was obtained for the use of buffy bags from healthy donors. Monocytes ($0.5.10^6$ cells/ml) were cultured in RPMI-1640 (Lonza, Verviers, Belgium) with 10% fetal calf serum (Life Technologies, Saint-Aubin, France), supplemented with GM-CSF (1000 IU/10^6 cells) and IL-4 (500 IU/10^6 cells) (Miltenyi Biotec) for 5 days to generate immature MoDCs. After 5 days, the differentiation of MoDCs (> 90% purity) was confirmed by flow cytometry upon loss of CD14 staining (M5E2 clone, BD Pharmingen, San Jose, CA, USA), expression of major histocompatibility complex (MHC) class II and CD1a (HI149 and G46.6 clones, respectively, BD Pharmingen). Acquisition was performed on a LSR II cytometer with FACSDiva software (version 6.1, BD Biosciences, Le Pont au Claix, France). Murine BMDCs were generated as described previously.[16] Briefly, bone marrow cells were extracted from FVIII exon 16 knock-out C57BL/6 mice and cultured in Petri dishes (2.10^6 cells/10 ml/plate) for 10 days in RPMI-1640 supplemented with 10% fetal calf serum, 50 mM 2-mercaptoethanol and 200 U/ml murine recombinant GM-CSF (Cellgenix Technology Transfer, Freiburg, Germany). Supplemented medium was replaced at days 3, 6 and 8. BMDCs purity and phenotype were validated by the expression of CD11c (HL3 clone, BD Pharmingen).

In vitro *FVIII uptake by immature MoDCs and BMDCs*

B domain-deleted FVIII (20 nM) was pre-incubated with equimolar concentrations of ESH8, BO2C11 or with a 2 molar excess BO2C11 Fab fragments for 30 min at 37°C. Samples were then incubated with 5-day-old immature MoDCs or with 9-day-old immature BMDCs ($0.2.10^6$ cells/100 µl) in Iscove's Modified Dulbecco's Medium for 30 min at 37°C or 4°C. Cells were washed with ice-cold phosphate buffered saline (PBS) and fixed with BD Cytofix™ Fixation buffer (BD Biosciences) for 20 min at 4°C. Cells were then permeabilized for 30 min at room temperature with permeabilization buffer (eBiosciences), and FVIII was stained using biotinylated GMA-8015 (2 µg/ml), followed by streptavidin-PE (1 µg/ml, BD Biosciences) for 30 min at room temperature. Cells were analyzed by flow cytometry. The uptake was quantified as the difference in median fluorescence intensities between 37°C and 4°C ($\Delta MFI_{37°C-4°C}$), to exclude the signal generated by the binding of FVIII to the cell surface.

In vitro *activation of a FVIII-specific HLA-DRB1*0101-restricted mouse $CD4^+$ T cell hybridoma*

Activation of the HLA-DRB1*0101-restricted mouse $CD4^+$ T cell hybridoma specific for human FVIII (1G8-A2), was assessed as previously described.[17] FVIII (10 nM) pre-incubated or not with 2 molar excess of BO2C11 was incubated with 10,000 MoDCs or 200,000 mitomycin C-treated mouse splenocytes from SUREL1 mice and co-cultured with 100,000 T cells in X-VIVO[15] medium (Life Technologies) for 18 hr at 37°C. Controls included T cells incubated alone, or incubated with MoDCs/BMDCs in the presence of concanavalin A (2 µg/ml, Sigma-Aldrich), or in the absence of FVIII. Levels of interleukin-2 (IL-2) secreted in the supernatant by T cells were assessed using the BD OptEIA™ mouse IL-2 ELISA set (BD Biosciences). Of note, MoDCs do not produce IL-2 when incubated with FVIII alone.[18]

Animals and administration of FVIII

Eight to 12 week-old FVIII exon 16 knock-out C57Bl/6 mice and VWF/FVIII exon 16 double knock-out C57Bl/6 mice (Professor H.H. Kazazian, University of Pennsylvania School of Medicine, Philadelphia, PA, USA, and Drs. C Denis and O Christophe, INSERM U770, Le Kremlin-Bicêtre, France, respectively), were injected intravenously once a week for 4 weeks with either: i) B-domain deleted FVIII (0.2 µg, Refacto, Pfizer) pre-incubated with equimolar amounts of $F(ab')_2$ fragments of an IgG4 isotype control

or BO2C11, ii) B-domain deleted FVIII^{Y1680C} (0.4 μg) alone or pre-incubated with Fab fragments of BO2C11, or iii) B-domain deleted FVIII^{HSQ}, FVIII^{C1} or FVIII$^{R2215-20A}$ (0.5 or 1 μg). The presence of endotoxins in the different recombinant FVIII was evaluated using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript, Piscataway, NJ, USA). The measured values were below the accepted threshold (i.e., <0.01 ng endotoxin/20 g mouse weight). Blood was collected on heparinized capillaries by retro-orbital bleeding 4 days after the fourth administration of FVIII. Plasma was collected and kept at -80°C until use. Mice were handled in agreement with French ethical authorities (authorization #23BA53).

Titration of anti-FVIII IgG and FVIII inhibitors

ELISA plates were coated with FVIII (1 μg/ml, Recombinate®, Baxter, Maurepas, France), in bicarbonate buffer pH 9.5, overnight

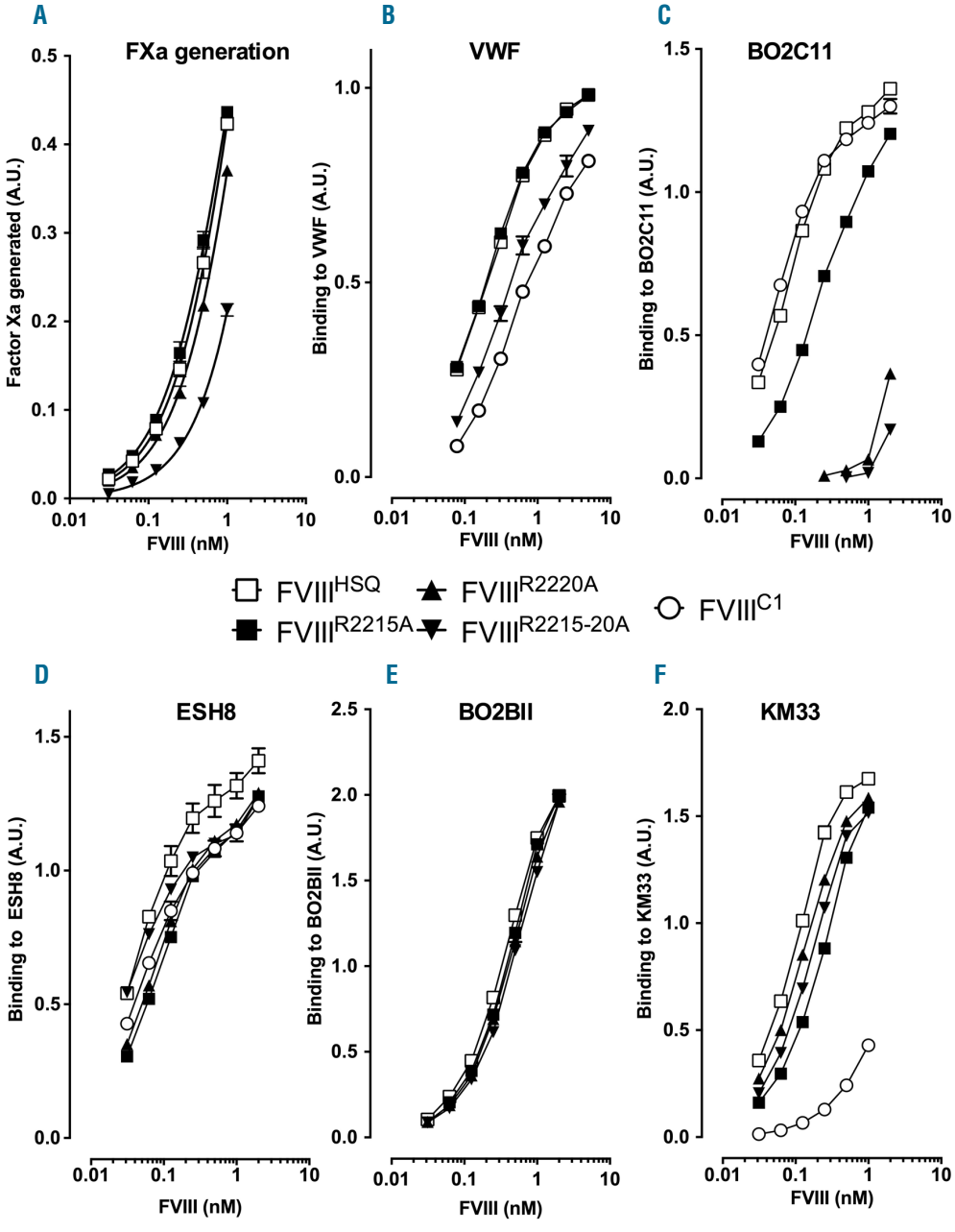

Figure 2. Characterization of FVIII containing alanine substitutions of the residues predicted to interact with BO2C11. Panel A. B domain-deleted wild-type FVIII (FVIII^{HSQ}), FVIII^{R2215A} or FVIII^{R2220A} were serially diluted 2-fold starting at 1 nM. Factor Xa generation was measured using a FVIII chromogenic assay. The reaction was stopped at 10 min using 20% acetic acid and the final absorbance measured at 405 nm. The data are represented as factor Xa generated in arbitrary units and are equivalent to the absorbance at 405 nm. VWF (Panel B), BO2C11 (human anti-C2 domain antibody, Panel C), ESH8 (mouse anti-C2 domain antibody that does not compete with BO2C11, Panel D), BO2BII (human anti-A2 domain antibody, Panel E) and KM33 (human anti-C1 domain antibody, Panel F) were immobilized on microtiter plates. After blocking, FVIII^{HSQ}, FVIII^{R2215A}, FVIII^{R2220A}, FVIII$^{R2215-20A}$ or FVIII^{C1} were serially diluted across the plates, and bound FVIII was revealed using either the biotinylated anti-A2 antibody, GMA-8015, or, in the case of BO2BII-bound FVIII, ESH8, followed by an anti-mouse-HRP antibody. The graphs are represented as absorbance measured at 492 nm (mean±SEM). FXa: factor Xa; VWF: von Willebrand factor; A.U.: arbitrary unit; FVIII: factor VIII.

at 4°C. After blocking with PBS, 0.05% Tween 20 and 2% BSA, the mouse plasma was incubated for 1 hr at 37°C. Serial dilutions of the samples were incubated for 1 hr at 37°C, and bound IgG were revealed using a horseradish peroxidase conjugated polyclonal goat anti-mouse IgG antibody (Southern Biotech) and the OPD substrate. Absorbance was read at 492 nm. The monoclonal mouse anti-human FVIII IgG mAb6 was used as a standard. Titers are expressed in arbitrary units (A.U.). Inhibitory titers were estimated by incubating heat-inactivated mouse plasma with human standard plasma (Siemens Healthcare Diagnostics) for 2 hr at 37°C. The residual FVIII procoagulant activity was measured using a chromogenic assay (Siemens Healthcare Diagnostics). Results are expressed in Bethesda units (BU)/ml that correspond to the reciprocal dilution of the mouse plasma that yielded 50% residual FVIII activity.

In vivo *clearance of wild-type and mutated FVIII*

FVIII exon 16 knock-out mice and VWF/FVIII exon 16 double knock-out C57Bl/6 mice were injected intravenously with wild-type and mutated FVIII (10 nM in 100 μl). Blood was collected in 0.129 M sodium citrate at different time points after FVIII administration. FVIII residual levels in mouse plasma were determined by sandwich ELISA using ESH8 and biotinylated GMA-8015 as capture and detection antibodies, as described above. Data are plotted as a percentage of the initial FVIII level, measured 5 minutes after FVIII infusion, *versus* time (mean±SEM). Values at 5 min post-injection did not differ between the groups of mice treated with the different FVIII. Experimental data was fitted with a one-phase exponential decay equation using GraphPad Prism software (version 6.01).

Results

FVIII bound to BO2C11 induces diminished immune responses in vivo

We first evaluated whether BO2C11, a human C2-specific anti-FVIII IgG, inhibits FVIII uptake by MoDCs or BMDCs *in vitro*. We hypothesized that the uptake is restricted within the BO2C11 binding region[14] and thus, as a control, employed another C2-specific antibody, ESH8. ESH8 does not bind to the BO2C11 epitope and, unlike BO2C11, does not inhibit FVIII binding to VWF or phosphatidylserine membrane surfaces.[19] We incubated 20 nM FVIII or FVIII pre-incubated with equimolar concentrations of anti-C2 antibodies prior to incubation with MoDCs or BMDCs. Additionally, we evaluated the inhibition caused by pre-incubating with a 2-fold molar excess of the Fab fragments of BO2C11. Following the addition of BO2C11 or the corresponding Fab fragments, we

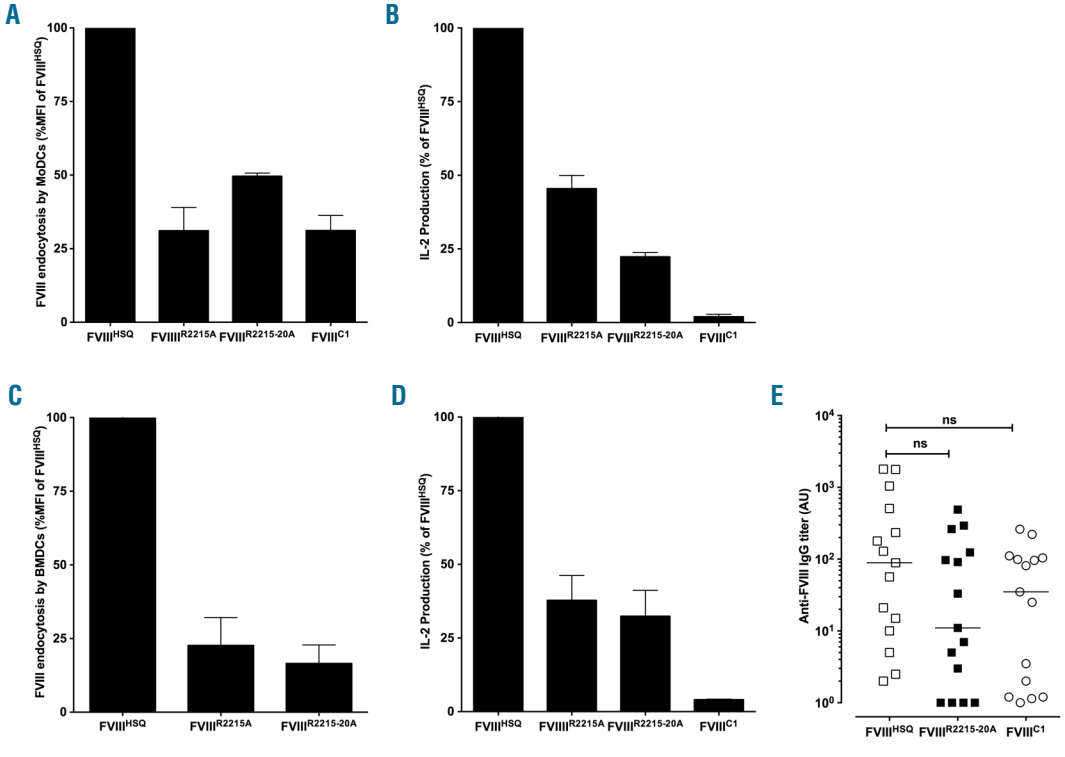

Figure 3. domain mutations alter FVIII endocytosis and presentation by APCs, but do not alter FVIII immunogenicity *in vivo*. B domain-deleted wild-type FVIII ($FVIII^{HSQ}$) or the mutants $FVIII^{R2215A}$, $FVIII^{R2215-20A}$ or $FVIII^{C1}$ were added at 20 nM to MoDCs (Panel A) or BMDCs (Panel C) for 30 min. Internalized FVIII was detected as described in Methods. Results are expressed as the percentage of MFI, whereby 100% corresponds to MFI obtained with $FVIII^{HSQ}$. Panels B and D represent activation of a FVIII-specific HLA-DRB1*0101-restricted T-cell hybridoma by B domain-deleted FVIII-loaded (10 nM) HLA-matched human MoDCs or splenocytes from SURE-L1 mice, respectively. Supernatant was collected after 24 hr and the IL-2 produced by the activated T cells was measured. Representative of three experiments (mean±SEM). Panel E. FVIII-deficient mice were injected intravenously once weekly for 4 weeks with 1 μg of B domain-deleted $FVIII^{HSQ}$, $FVIII^{C1}$ or $FVIII^{R2215-20A}$. One week after the last injection, blood samples were collected. Anti-FVIII IgG titers are defined as arbitrary units based on standard curves generated using mAb6. Statistical significances were assessed using the two-tailed nonparametric Mann-Whitney U test. ns: not significant; FVIII: factor VIII; MoDCs: monocyte-derived dendritic cells; A.U.: arbitrary unit; IL-2: interleukin-2; IgG: immunoglobulin G; MFI: median fluorescence intensity; BMDCs: bone marrow-derived dendritic cells.

observed a more than 70% reduction in FVIII internalization by MoDCs (Figure 1A), and a reduction of about 30% in the case of BMDCs (*Online Supplementary Figure 2A*). The addition of ESH8 did not reduce FVIII uptake by MoDCs. Similarly, BO2C11 or Fab of BO2C11 inhibited FVIII presentation to a HLA-DR1-restricted CD4$^+$ T-cell hybridoma by more than 80% in the case of both MoDCs (Figure 1B) and splenocytes purified from HLA-DR1 Tg SURE-L1 mice (*Online Supplementary Figure S2B*), used as sources of APCs. Together, our results implicate a role for C2 membrane-binding residues in FVIII uptake by APCs. We next investigated the effect of BO2C11 on FVIII immunogenicity *in vivo*. We administered FVIII pre-incubated with F(ab')$_2$ of an isotype control or of BO2C11 for 4 weeks at weekly intervals. After 4 weeks, BO2C11-bound FVIII exhibited diminished immunogenicity compared to the isotype control-treated mice (Figure 1C).

Characterization of FVIII variants mutated in the BO2C11 epitope

We generated FVIII variants, wherein the two arginine residues located at position 2215 and 2220 that belong to the BO2C11 epitope, are mutated to alanine residues. The purified FVIII mutants exhibited specific activities between 4800-8000 IU/mg (Figure 2A) similar to that of non-mutated FVIII (FVIII^{HSQ}). Substitutions at either R2215 or R2220 did not alter binding to VWF, while substitution of both R2215 and R2220 lead to a marginally reduced binding to VWF (Figure 2B). Substitutions at R2215 or R2220 resulted in diminished binding to BO2C11 (Figure 2C), with substitution at R2220 resulting in a near complete inhibition of FVIII interaction with BO2C11. Double mutation of these residues (FVIII$^{R2215\text{-}20A}$) provided little additional benefit, confirming that R2220 contributes to most of the binding to BO2C11. Importantly, these FVIII

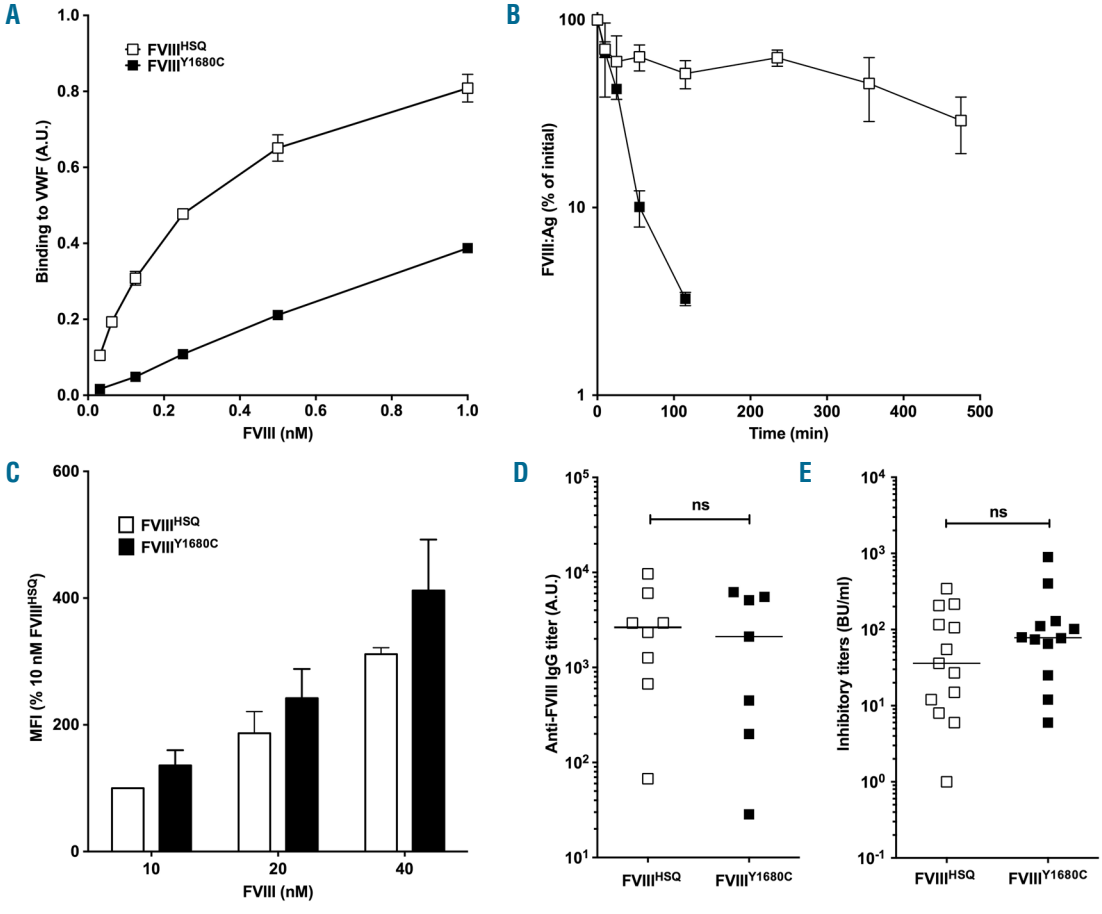

Figure 4. FVIII binding to VWF does not alter endocytosis in vitro or immunogenicity *in vivo*. Panel A. The binding of B domain-deleted FVIII^{HSQ} and FVIII^{Y1680C} to VWF was studied using an ELISA as described in the Methods. Results (mean±SEM) are expressed in arbitrary units. Panel B. FVIII^{HSQ} (10 nM, open squares) or FVIII^{Y1680C} (closed squares) in 100 µl were administered into FVIII-deficient mice and the residual FVIII levels were measured at different time points (n=3 mice per time point) by ELISA (FVIII:Ag). The data is plotted as a percentage of the initial FVIII level (measured 5 minutes after administration, mean±SEM) *versus* time. Panel C. FVIII^{HSQ} or mutants FVIII^{Y1680C} were added at increasing concentrations (10, 20 and 40 nM) to immature MoDCs (n=3 different donors) for 30 min at 37°C in serum-free medium and in the absence of VWF. Cells were subsequently washed, fixed, permeabilized and stained for FVIII using a FITC-labeled anti-A2 domain-specific antibody followed by fluorescence-activated cell sorting (FACS). Data are expressed as the percentage of MFI (mean±SEM), whereby 100% corresponds to the MFI observed for 10 nM of FVIII^{HSQ}. Panels D and E. FVIII-deficient mice were administered with FVIII^{HSQ} (0.5 µg, open squares) or FVIII^{Y1680C} (0.5 µg, closed squares), intravenously once a week for 4 weeks. Anti-FVIII IgG titers were measured using an ELISA as described above (D). Inhibitory titers towards FVIII were assessed using a modified Bethesda assay (E). Horizontal bars depict medians. The statistical significance was assessed using the two-tailed non-parametric Mann-Whitney U test. ns: not significant; FVIII: factor VIII; VWF: von Willebrand factor; A.U.: arbitrary unit; MFI: median fluorescence intensity; IgG: immunoglobulin G; Ag: antigen.

variants retained binding to antibodies targeting other domains of FVIII. In particular, the mutations did not have significant effects on the ability of FVIII to interact with ESH8 (mouse anti-C2 IgG), BO2BII (human anti-A2 IgG) or KM33 (human anti-C1 IgG, Figure 2D-F). Recently, the three residues R2090A/F2092A/K2093A in the FVIII C1 domain that belong to the KM33 epitope, were implicated in FVIII uptake.[11] Hence, as a control, we generated the R2090A/F2092A/K2093A FVIII mutant, referred to as "FVIII^{C1}". As expected, FVIII^{C1} showed drastically reduced binding to KM33 (Figure 2F), and unaltered binding to BO2C11 and ESH8 (Figure 2C-D).

C2 domain residues are implicated in FVIII uptake by APCs

We next evaluated the role of C2 residues in FVIII uptake and presentation. Human MoDCs and murine BMDCs were incubated with 20 nM FVIII, fixed and permeabilized, and FVIII was revealed with the anti-A2 antibody GMA-8015. FVIII uptake was dose-dependent (*Online Supplementary Figure S3*) and significantly reduced for FVIII^{R2215A} and FVIII$^{R2215\text{-}20A}$ (Figure 3A and 3C). We confirmed our observation in an antigen presentation assay to a HLA-DR1-restricted CD4$^+$ T-cell hybridoma using MoDCs derived from a HLA-DR1 donor, and using splenocytes from HLA-DR1 transgenic SURE-L1 mice (Figure 3B and 3D). Importantly, the epitope of CD4$^+$ T-cell hybridoma is located in the 2004-2031 amino acid stretch at the A3-C1 junction of FVIII (*data not shown*); it is therefore distant from the mutated residues, thus ruling out the fact that the lack of T-cell activation is consecutive to a disruption of the T-cell epitope. We observed a significant decrease in FVIII presentation by human and mouse dendritic cells (DCs), as measured by reduced IL-2 secretion in the case of both FVIII^{R2215A} and FVIII$^{R2215A\text{-}R20A}$. Our present data indicate that the C2 domain participates in FVIII uptake and involves at least R2215.

To further evaluate the importance of the C2 mutants in *in vivo* immunogenic uptake, we compared the immunogenicity of the different FVIII variants in FVIII-deficient mice. Cage-matched siblings were administered intravenously 4 times weekly with 1 μg of FVIII^{HSQ}, FVIII$^{R2215\text{-}20A}$ or FVIII^{C1}, used as a control. In contrast to previous reports,[11] FVIII-deficient mice generated an antibody response not only to FVIII^{HSQ} but also to FVIII^{C1} (Figure 3E). In addition, FVIII$^{R2215\text{-}20A}$ also presented with the same immunogenicity as FVIII^{HSQ}. The data suggest that mutating residues in the C1 and C2 domains of FVIII, at least in our hands, has no effect on FVIII immunogenicity in FVIII-deficient mice *in vivo*.

The immunogenicity of FVIII is independent from its ability to interact with endogenous VWF

Because BO2C11 inhibits FVIII interaction with VWF, we investigated the possibility that FVIII immunogenicity in the presence of BO2C11 (Figure 1C) was diminished due to its faster clearance. To address this potential bias, we generated a FVIII variant, FVIII^{Y1680C}, which cannot associate with VWF. The specific activities of FVIII^{HSQ} and FVIII^{Y1680C} were similar as assessed by one-stage and chromogenic assay (7300±165 and 6300±185 IU/mg, respectively, mean±SD). FVIII^{Y1680C} exhibited a significant reduction in its ability to interact with VWF by ELISA (Figure 4A). Accordingly, FVIII^{Y1680C} exhibited a rapid clearance in FVIII-deficient mice with a $t_{1/2}$ of approximately 20 minutes (Figure 4B). We also confirmed that the Y1680C mutation does not alter the *in vitro* endocytosis of FVIII by MoDCs (Figure 4C). Next, we performed comparative immunogenicity studies of FVIII^{HSQ} and FVIII^{Y1680C} using FVIII-deficient mice. Remarkably, FVIII^{Y1680C} was as immunogenic as FVIII^{HSQ} (Figure 4D). The inhibitor titers were also not significantly different (Figure 4E). In addition, the immunogenicity of FVIII^{Y1680C} in FVIII-deficient mice was similar to that of FVIII^{HSQ} in double FVIII/VWF-deficient mice (*data not shown*).

C domain mutations exhibit reduced immunogenicity only in the absence of VWF

Having demonstrated that the ability of FVIII to interact with endogenous VWF does not modulate the magnitude of the anti-FVIII immune response, we investigated the immune protective effect of BO2C11 using FVIII^{Y1680C}. FVIII^{Y1680C} alone or pre-incubated with a 2 molar excess of BO2C11 Fab fragments was administered to FVIII-deficient mice at weekly intervals for 4 weeks. Five days after the last FVIII administration, the anti-FVIII immune response was measured. We observed a significant reduction both in the anti-FVIII antibody response (Figure 5A), and FVIII-specific T-cell proliferation (*Online Supplementary Figure S4*), when FVIII^{Y1680C} was pre-incubated with BO2C11 Fab fragments. In contrast, in double FVIII/VWF-deficient mice, the immunogenicity of FVIII$^{R2215\text{-}20A}$ (1300±303 μg/ml, 90±17 BU/ml, mean±SEM) was not statistically different from that of FVIII^{HSQ} (2258±596 μg/ml, 180±62 BU/ml, Figure 5C,D, P>0.2), despite an increased residence time in the circulation of FVIII$^{R2215\text{-}20A}$ (Figure 5B, 41 min [95% confidence interval (CI): 39-51]) as compared to FVIII^{HSQ} (17 min [13-23]). Conversely, FVIII^{C1} showed a statistically significant reduction in immunogenicity in double FVIII/VWF-deficient mice as compared to FVIII^{HSQ}, both in terms of levels of anti-FVIII IgG (460±144 and 2258±596 μg/ml, respectively; P<0.001) and inhibitory titers (38±17 and 180±62 BU/ml, respectively; P<0.05). Similar observations were obtained with a FVIII mutant that combined the triple C1 mutation and a replacement of the R2215 residue with a serine (*Online Supplementary Figure S5*).

Discussion

The endocytosis of an antigen by APCs is not sufficient for the induction of a primary CD4$^+$ T-cell-dependent immune response. It is however a prerequisite to its presentation to naïve antigen-specific CD4$^+$ T cells. Over the years, several receptors have been identified for FVIII[5,6,20,21] that have mostly been implicated in FVIII catabolism.[8,22] To date, the only receptor incriminated in FVIII internalization by APCs is the C-type lectin receptor CD206, that binds mannose-ending glycans linked to Asn239 in the A1 domain of FVIII, and Asn2118 in the C1 domain.[7] The importance of the C1 domain of FVIII in binding to phospholipids and participating in FVIII endocytosis has recently been proposed. Thus, Lys 2092 and Phe 2093 in C1 were shown to participate in binding to phosphatidylserine and to platelets.[23,24] Interestingly, co-incubation of FVIII with KM33, a monoclonal anti-C1 IgG that targets the R2090, K2092 and F2093 residues, prior to injection into FVIII-deficient mice, was associated with a decrease in FVIII immunogenicity.[10] Additionally, a triple

R2090A/K2092A/F2093A FVIII mutant demonstrated reduced endocytosis by MoDCs and macrophages, and reduced immunogenicity in FVIII-deficient mice.[11] Because the C2 domain of FVIII shares structural homology with the C1 domain,[25] and is a major membrane-binding motif[26,27] containing several charged/basic residues, we investigated whether it also participates in the endocytic process by APCs, that leads to FVIII processing and presentation to $CD4^+$ T cells. Our results demonstrate that masking part of the C2 domain with BO2C11, a human monoclonal IgG that interacts with solvent-exposed basic and hydrophobic side chains on the C2 membrane-binding loops,[14] reduces FVIII endocytosis by MoDCs and presentation to T cells *in vitro*, as well as immunogenicity in mice. The protective effect of BO2C11 was epitope-specific, since ESH8, a non-overlapping mouse monoclonal anti-C2 IgG, failed to block FVIII uptake *in vitro*. ESH8 in fact increased the uptake process. Since the binding of ESH8 to FVIII was proposed to prevent a conformational change in the C2 domain of the FVIII light chain,[28] our

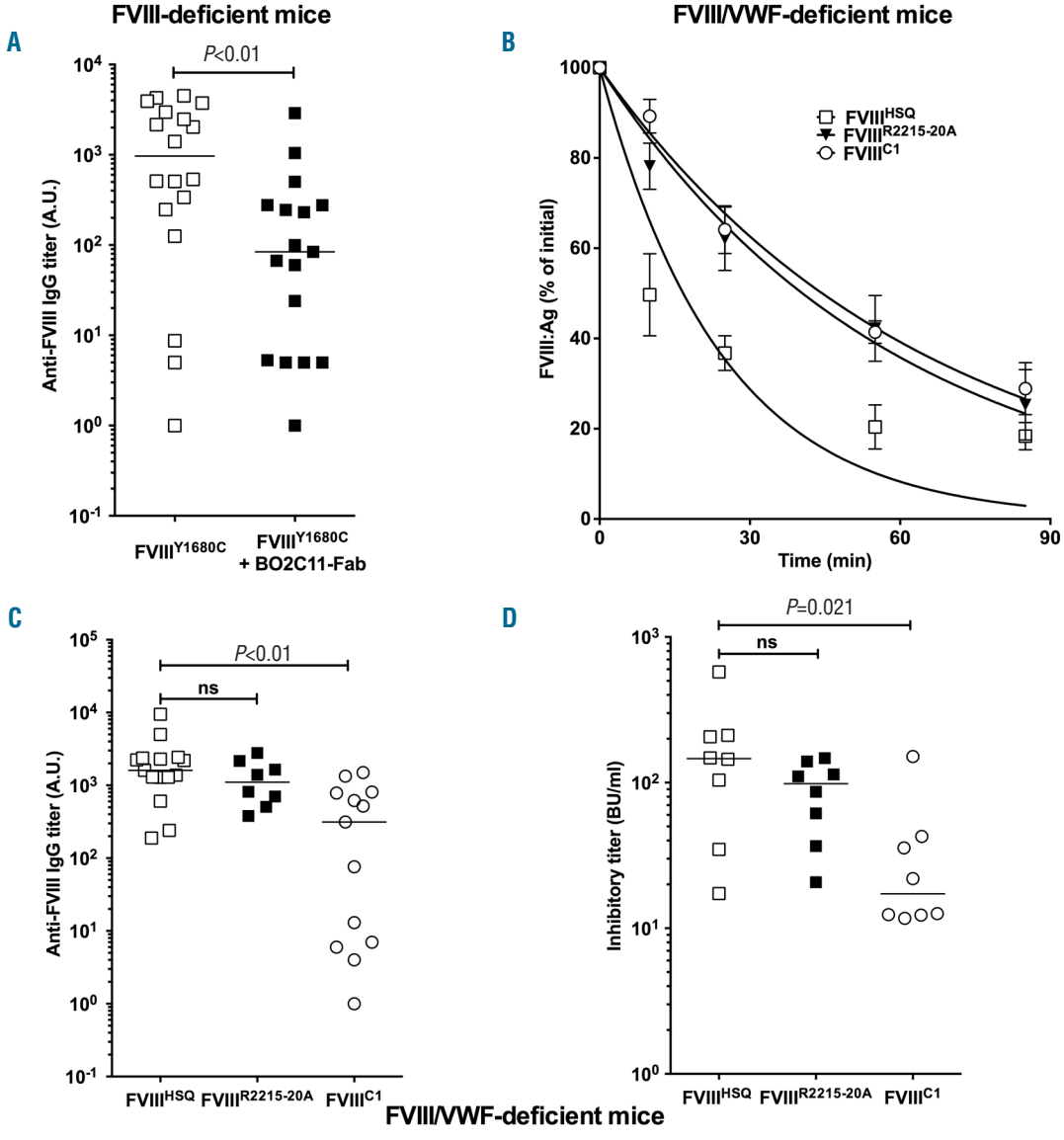

Figure 5. Contribution of the C1 and C2 domains of FVIII to FVIII immunogenicity in the absence of binding to VWF. Panel A. FVIII-deficient mice were administered with B domain-deleted $FVIII^{Y1680C}$ (0.4 μg, open squares) or $FVIII^{Y1680C}$ pre-incubated with a 2-fold molar excess of BO2C11 Fab (closed squares) intravenously once a week for 4 weeks. One week after the last injection, blood samples were collected. Anti-FVIII IgG titers are defined as arbitrary units based on standard curves generated using mAb6. The graph depicts a pool of two independent experiments. Panel B. B domain-deleted $FVIII^{HSQ}$, $FVIII^{C1}$ or $FVIII^{R2215-20A}$ (10 nM in 100 μl) were administered to FVIII-deficient mice and the residual FVIII levels were measured at different time points (n=3 mice per time point) by ELISA. The data are plotted as a percentage of the initial FVIII levels (measured 5 minutes after administration) *versus* time (mean±SEM) and are representative of 2 independent experiments. Experimental data were fitted using a one-phase decay curve to determine the *in vivo* half-lives. Panels C and D. Double FVIII/VWF-deficient mice were injected intravenously once a week for 4 weeks with 0.5 μg of $FVIII^{HSQ}$, $FVIII^{C1}$ or $FVIII^{R2215-20A}$. One week after the last injection, blood samples were collected. Anti-FVIII IgG titers were measured as described above (C). Inhibitory titers towards FVIII were assessed using a modified Bethesda assay (D). Horizontal bars depict medians. Statistical significances were assessed using the two-tailed non-parametric Mann-Whitney U test. ns: not significant: FVIII: factor VIII; IgG: immunoglobulin G; A.U.: arbitrary unit; Fab: fragment antigen binding; VWF: von Willebrand factor; Ag: antigen.

observation may partly relate to a particular conformation of ESH8-bound FVIII, that orients FVIII to facilitate the uptake process. Interestingly, the mutation of key charged residues on C2 that belong to the BO2C11 epitope, resumed the effect of BO2C11 on FVIII endocytosis and presentation *in vitro*. Our results are thus reminiscent of the aforementioned findings on the role played by the FVIII C1 domain in immunogenic FVIII uptake. Together, the data demonstrate that both the C1 and C2 domains of FVIII play a role in FVIII endocytosis by MoDCs. Since lactadherin, a potent competitor for phosphatidylserine binding, does not inhibit FVIII endocytosis by MoDCs,[11] FVIII uptake is probably independent of binding to phospholipids. Rather, we speculate that FVIII uptake in the absence of VWF *in vitro* involves charged interactions with a putative highly negatively charged receptor, or a potential role for co-receptors, such as heparan sulfate proteoglycans. The respective contribution, either redundant, additive or synergistic, to FVIII internalization of residues in the C1 and C2 domains, and of mannose-ending N-linked glycans in the A1 and C1 domains, as well as the sequence of events that allows FVIII binding at the cell surface and internalization, remain to be deciphered.

While co-incubation of FVIII prior to injection into FVIII-deficient mice, with BO2C11 or with KM33[10] reduced the anti-FVIII IgG response, neither the $FVIII^{R2215\text{-}20A}$ C2 mutant nor the triple $FVIII^{C1}$ mutant demonstrated reduced immunogenicity in FVIII-deficient mice in our hands. These observations may be explained either by a steric hindrance of the C domains of FVIII by the antigen-binding domains of BO2C11/KM33, or by the implication in FVIII internalization of additional amino acids within the BO2C11 epitope on C2, or within the KM33 epitope on C1. Alternatively, the findings may be accounted for by an interfering effect of endogenous VWF. Indeed, the T2086-S2095 region of the C1 domain was recently shown to be buried upon association with VWF,[29] and the triple $FVIII^{C1}$ mutant retains the ability to interact with VWF. In support of this, in the absence of endogenous VWF, the triple $FVIII^{C1}$ mutant showed reduced immunogenicity as compared to $FVIII^{HSQ}$ *in vivo*. In contrast, the $FVIII^{R2215\text{-}20A}$ C2 mutant, that also retains most of VWF binding and demonstrates reduced endocytosis *in vitro*, was as immunogenic as $FVIII^{HSQ}$ in mice lacking VWF. Of note, the lack of reduced immunogenicity of the triple $FVIII^{C1}$ mutant in FVIII-deficient mice is at odds with the report by Wroblewska *et al.*;[11] it remains unclear whether the observed difference may relate to differences in levels of VWF in different strains of mice.[30] Although the C2 domain has been identified as an essential membrane interactive motif, with the C1 domain providing additional membrane binding affinity,[23,26,27] the results suggest that C2 residues do not play a predominant role in immunogenic FVIII in the absence of endogenous VWF. Instead, we speculate that the *in vivo* role in endocytosis of the targeted R2215 and R2220 basic residues in the C2 domain is only secondary to that of other membrane accessible residues within the BO2C11 epitope. This is supported by the observation that the pre-incubation of the $FVIII^{Y1680C}$ mutant, which does not bind VWF, with BO2C11, reduced its immunogenicity in FVIII-deficient mice. Additional mutational analysis of membrane accessible hydrophobic residues within the BO2C11 epitope shall shed light on the specific residues of the C2 domain of FVIII that are implicated in the *in vivo* immunogenic uptake process.

VWF has controversially been proposed to reduce the immunogenicity of therapeutic FVIII.[31] Unexpectedly, the present results indicate that the presence of endogenous VWF, or the capacity of exogenous FVIII to bind endogenous VWF, does not alter the immunogenicity of FVIII in mice. Indeed, FVIII presented with the same degree of immunogenicity in FVIII-deficient mice and in double FVIII/VWF-deficient mice. Likewise, $FVIII^{Y1680C}$ with impaired binding to VWF induced similar anti-FVIII IgG levels as native FVIII in FVIII-deficient mice. The potential immunomodulatory role of VWF towards FVIII has been suggested by a suspected reduced prevalence of FVIII inhibitors in hemophilia A patients treated with exogenous VWF-containing plasma FVIII concentrates, as compared to patients receiving recombinant or highly purified products.[32,33] Controversial results have, however, been obtained upon studying large retrospective and prospective patient cohorts.[34,35] In parallel, studies performed in pre-clinical mouse models of hemophilia A have indicated that pre-incubation of recombinant FVIII with exogenous VWF leads to a reduction in the levels of inhibitory anti-FVIII antibodies following administration to mice,[36-39] although contradictory results have occasionally been generated.[40] *In vitro*, a protective role for VWF on FVIII endocytosis by human MoDCs was clearly shown,[10,41] although presentation of processed FVIII-derived peptides could still be detected, at least *in vitro*.[42] In addition, under shear stress, VWF fails to block FVIII internalization by macrophages, and both VWF and FVIII co-localize within the same cells.[43] This is in agreement with the observation that exogenous FVIII and exogenous VWF may be co-detected in splenic macrophages after injection into double FVIII/VWF-deficient mice.[44] The internalization of FVIII and VWF was, however, inhibited by the LRP antagonist receptor-associated protein (RAP) in the case of macrophages under shear stress, but not in the case of MoDCs in static conditions,[8] suggesting that this process is more relevant to FVIII catabolism than immunogenicity. Interestingly, the intricate role played by VWF in FVIII immunogenicity is reminiscent of its complex role towards FVIII catabolism. Indeed, binding of FVIII to VWF dictates FVIII residence time in the circulation, as shown in patients with type 3 von Willebrand disease.[45] Conversely, VWF-binding may mediate, at least in part, the catabolism of therapeutic FVIII,[20,46] which is further suggested by the fact that the half-life of modified Fc-fused or PEGylated FVIII barely exceeds that of VWF.[47,48]

Taken together, the results indicate that residues in the C1 and/or C2 domains of FVIII are implicated in immunogenic FVIII uptake, at least *in vitro*. Conversely, *in vivo*, the binding to endogenous VWF masks the reducing effect of mutations in the C domains on FVIII immunogenicity.

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