Regulation of immune responses to therapeutic factor VIII by transplacental delivery of Fc-fused immunodominant factor VIII domains or peptides

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Abstract

Patients with severe hemophilia A (HA) often develop undesired immune responses to therapeutic factor VIII (FVIII) that hamper replacement therapy with FVIII-derived products. The transplacental delivery of two Fc-fused FVIII domains in pregnant HA mice was shown to induce partial FVIII-specific immune tolerance in the offspring. Here, we evaluated whether the transplacental delivery of Fc-fused FVIII (rFVIIIFc) induces complete immune tolerance towards FVIII. rFVIIIFc injected to pregnant HA mice was poorly transferred to the fetal circulation and failed to confer tolerance to exogenous FVIII in the offspring. The poor transplacental delivery of rFVIIIFc was associated with the large size of the molecule and with the presence of positive patches at the surface of FVIII. It was, however, independent from the capacity of rFVIIIFc to bind Fcγ receptors or von Willebrand factor in the maternal circulation. Conversely, the transplacental delivery of Fc-fused A2 and C2 immunodominant domains of FVIII, as well as of Fc-fused molecules containing seven different immunodominant FVIII-derived peptides decreased the levels of anti-FVIII antibodies following FVIII replacement therapy in the offspring. Our study paves the way towards the development of engineered Fc-fused molecules able to efficiently cross the placenta and confer potent and long-lasting immune tolerance to protein therapeutics.

Introduction

Protective immunity is transferred from mothers to babies when maternal IgG cross the placenta. The transplacental delivery of maternal IgG involves the binding of their Fc fragment to the neonatal Fc receptor (FcRn) following endocytosis by the syncytiotrophoblasts, placental fetal endothelial cells, and by placental macrophages via non-specific fluid phase transport. Once inside the early acidic endosomes of the cells, the protonated imidazole side chains of histidine residues H310, H433 and H435 in the Fc fragment bind to the negatively charged E115, E116, D130 and E133 residues of FcRn,[1–3] which rescues the IgG from lysosomal degradation. The FcRn-IgG complex is then transported to the surface of the cell layer on the fetal side. After return to physiological neutral pH in fetal blood, the histidine residues in the Fc fragment are deprotonated, IgG dissociate from the FcRn and are released into the fetal blood.[4] In humans, the transfer starts in the second trimester of pregnancy with a peak at 36 weeks of gestation,[5] while in mice, a significant level of transcytosis of IgG from mothers to fetuses starts by the 15th day of gestation.[6]

The co-habitation of self and maternal antigens during pregnancy imposes a tolerogenic state on the immune systems of both the mother and fetus.[7] Through tissue transplantation experiments, Medawar and collaborators demonstrated for the first time that non-self antigens can be recognized as self by the immune system if presented early in life.[8] Previous research from our laboratory showed that Fc-fused antigens can be transferred from the blood of the mother to that of the fetus in an FcRn-dependent manner. On the fetal side, the administered Fc-fused antigens reached the lymphoid organs, were captured by antigen-presenting cells, which promoted the development of thymic and peripheral antigen (Ag)-specific regulatory T cells (Tregs).[9,10]

Hemophilia A (HA) is a rare bleeding disorder resulting from a missing or defective coagulation factor VIII (FVIII). Most frequently, HA patients are treated with recombinant or plasma-derived FVIII products to alleviate bleeding.[11] A significant number of HA patients develops FVIII neutralizing antibodies, called 'inhibitors', which hamper the efficacy of the treatment.[12] To date, the only option to decrease the levels of inhibitors is a prolonged exposure of their immune system to the protein, a protocol referred to as immune tolerance induction (ITI). But ITI is expensive,[13] inconvenient from a practical point of view, and fails in 20-30% of the patients.[14] In an HA mouse model, the injection of pregnant mice with the immunodominant domains of FVIII, A2 and C2, fused to the Fc domain of a mouse IgG1, conferred FVIII-specific tolerance to the progeny. The offspring of the treated mothers were thus partially protected from the development of neutralizing antibodies following administration of therapeutic FVIII.[10] Interestingly, while the A2 and C2 domains represent 20% of the entire FVIII protein, the offspring experienced a 10-fold reduction in levels of neutralizing anti-FVIII IgG.

In the present study, we investigated whether the materno-fetal transfer of FVIII fused to the Fc fragment of human IgG1 (rFVIIIFc) may impose complete tolerance towards therapeutic FVIII in the offspring. rFVIIIFc was poorly transferred and failed to induce tolerance in the progeny of rFVIIIFc-treated HA mice. The size of the molecule and the net positive charge of the FVIII light chain (LCh) contributed to the impaired transplacental delivery of rFVIIIFc. In contrast, the administration to pregnant HA mice of human Fc-fused molecules (either A2/C2 domains or immunodominant FVIII peptides) that were efficiently transplacentally delivered reduced the anti-FVIII immune response in the offspring following challenge with exogenous therapeutic FVIII.

Methods

Sources of Fc-fused factor VIII and factor IX

Fc-fused factor IX (FIX) (Alprolix®) as well as the mutants rFVIIIFc^{N297A} with impaired binding for Fcγ receptors (FcγR)[15] (*Online Supplementary Figure S1A*) and rFVIII^{Y1680F}Fc unable to bind von Willebrand factor (vWF)[16] (*Online Supplementary Figure S1B*) were kind gifts from Sanofi-Genentech. The rFVIIIFc variants, Fc-fused FVIII domains and Fc-fused FVIII-derived immunodominant peptides were obtained as described in the *Online Supplementary Methods*.

Mice

Factor VIII exon 16 knock-out (HA) mice[17] and double FVIII/VWF-KO (DKO) mice on the C57BL/6 background were used. Animals were handled in agreement with local ethical authorities (approved by the Charles Darwin ethics committee, authorizations APAFIS #31752-2021051916032241).

Evaluation of transplacental delivery

Pregnant HA mice were injected intravenously (iv) at day 17.5 of gestation with similar molar amounts (170-225 pmoles) of the different molecules (FVIII, IgG1, as well as Fc-fused FVIII, FVIII domain, FVIII peptides or FIX). Blood from pregnant mice was collected 5 minutes (min) and 4 hours (hr) after injection. Fetuses were recovered at 4 hr, and blood was collected. The plasma from 2-3 fetuses was pooled and all plasma samples were kept at -80°C until analysis. Molecules were quantified in plasma by ELISA (*Online Supplementary Appendix*).

Percentage of transfer of the molecules was calculated as: (amounts in fetuses' blood at 4 hr / amount in mothers' blood at 5 min) x 100, using blood volumes of 2 mL and 100 μL for the mothers and fetuses, respectively.[18–20]

For imaging experiments, the molecules were conjugated to Alexa Fluor 680 and injected iv to pregnant HA mice at day 17.5 of gestation. Four hours later, the mothers' organs and fetuses were collected for fluorescence imaging on an IVIS LUMINA II (Caliper Life Sciences, Hopkinton, MA, USA).

Statistical analysis

Data were analyzed using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA). The normal (Gaussian) distribution of data was tested using the Shapiro-Wilk test. Differences were statistically assessed using the two-tailed Mann-Whitney test, the Kruskal-Wallis test or with one-way ANOVA. Multiple comparisons were performed when indicated. All box and whiskers in the graphs depict medians with hinges as 5th to 75th percentiles, as well as the minimum and maximum values.

Results

Treatment of pregnant hemophilia A mice with A2Fc and C2Fc, but not factor VIII fused to the Fc fragment of human IgG1, modulates the immune response towards factor VIII in the offspring

We first validated the transplacental delivery of rFVIIIFc. Injection of 225 pmoles of rFVIIIFc (50 μg) to E17.5 pregnant HA mice allowed the detection of FVIII protein (mean ±Standard Deviation [SD]: 0.03±0.01 nM) (Figure 1A) in the fetuses 4 hr later. Accordingly, FVIII procoagulant activity was detected after the injection of rFVIIIFc (0.09±0.08 IU/mL at 3 hr and 0.04±0.04 IU/mL at 4 hr) (Figure 1B, *Online Supplementary Figure S2*), confirming the structural integrity of FVIII following transcytosis. rFVIIIFc was transplacentally delivered in a dose- and time-dependent manner (Figure 1A). Of note, rFVIIIFc was not detected in the fetuses' plasma 24 hr after injection (*data not shown*). Therapeutic B domain deleted (BDD)-FVIII devoid of Fc-fused fragment, used as control, failed to be transferred from the maternal to the fetal circulation (Figure 1A), indicating dependency of the transplacental transfer on the FcRn. We then investigated

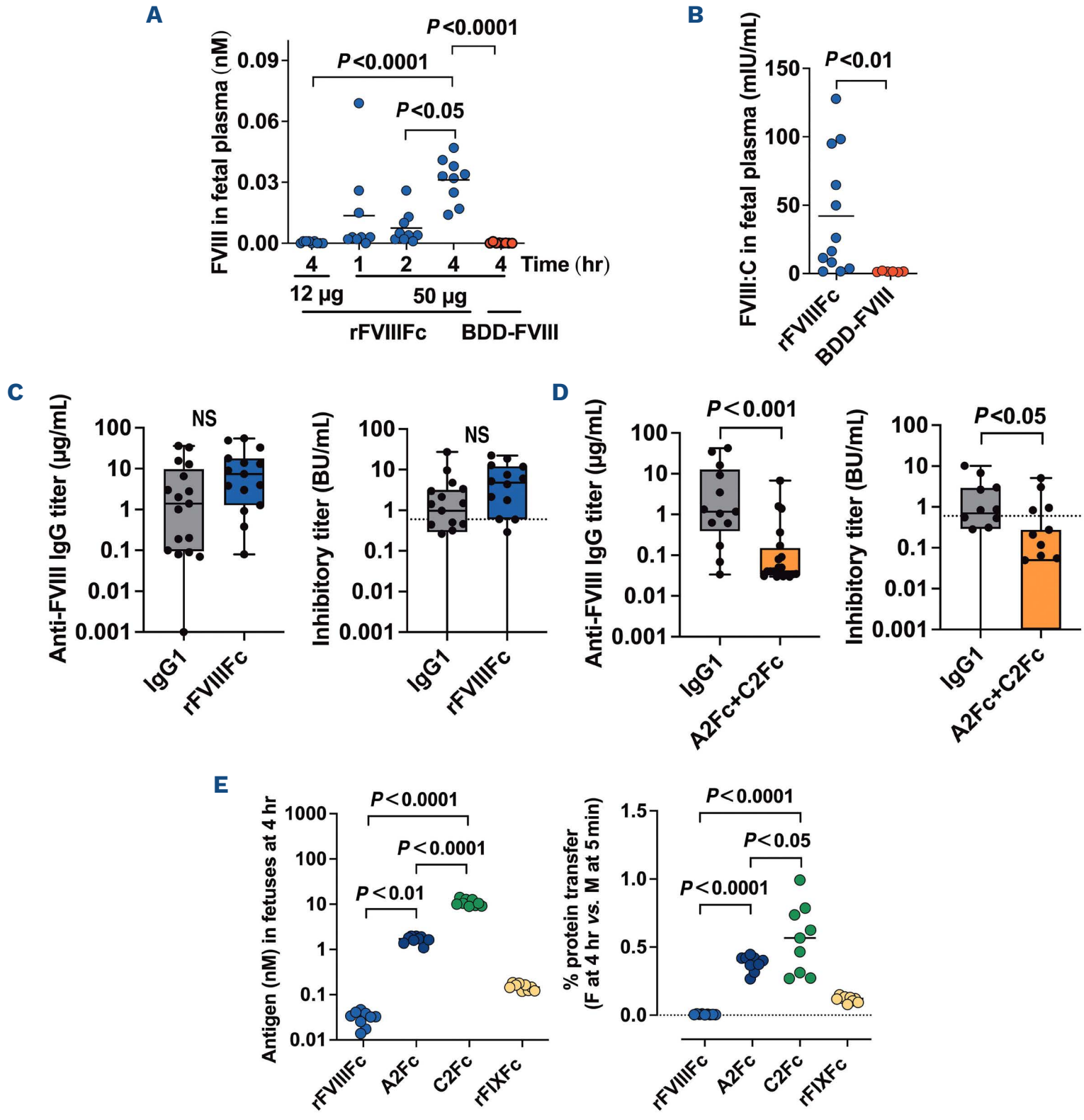

Figure 1. Injection of pregnant hemophilia A mice with A2Fc and C2Fc, but not FVIII fused to the Fc fragment of human IgG1, reduces the immune response towards factor VIII in the offspring. (A) Naïve pregnant hemophilia A (HA) mice (N=3) were injected intravenously (iv) at day 17.5 of gestation with 12 μg (57 pmoles) or 50 μg (225 pmoles) of factor VIII (FVIII) fused to the Fc fragment of human IgG1 (rFVIIIFc), or with 50 μg (294 pmoles) of BDD-FVIII. The graph depicts the concentration of the FVIII in fetuses' plasma obtained 1, 2 or 4 hours (hr) after injection to the mothers, measured by ELISA and expressed in nanomolar concentrations. (B) The graph depicts the activity of FVIII (FVIII:C) measured in the fetuses' plasma 4 hr after injection of 50 μg of BDD-FVIII or rFVIIIFc to day 17.5-pregnant HA mice, using a one-stage clotting assay. In (A) and (B), each dot represents a pool of plasma from 2 to 3 fetuses. Horizontal lines depict the means. (C and D) The humanized monoclonal IgG1, trastuzumab (Herceptin®) (1,500 pmoles / 225 μg) or the indicated Fc-fused proteins (225 pmoles / 50 μg rFVIIIFc, 1,500 pmoles / 144 μg A2Fc, 1,500 pmoles / 105 μg C2Fc) were injected into pregnant HA mice at E16, E17 and E18 of gestation. The 4-6-week old progeny was injected weekly with BDD-FVIII (1 μg/mouse). After 4 (C) or 5 (D) injections, blood samples were collected and anti-FVIII IgG titers were quantified by ELISA. Results are expressed in μg/mL (left). Inhibitory titers toward FVIII were quantified by chromogenic FVIII assay (right). Each dot represents an individual mouse. (Right) The dotted lines represent the limit of detection of inhibitory IgG, i.e., 0.6 BU/mL. (E) Comparison of the transplacental delivery of rFVIIIFc in naïve pregnant HA mice (N=3) injected iv at day 17.5 of gestation with rFVIIIFc (225 pmoles), A2Fc or C2Fc (170 pmoles) or rFIXFc (225 pmoles). The graph depicts the concentration of the antigens (measured by ELISA) in fetuses (F) plasma obtained 4 hr after injection to the mothers (M), expressed in nanomolar concentration (left) or expressed as percentage of antigen detected in the fetuses' plasma at 4 hr *versus* that detected in mothers' plasma at 5 minutes (min) (right, defined as percentage of protein transfer). Each dot represents a pool of plasma from 2 to 3 fetuses. Horizontal lines depict means. Differences were statistically assessed using the Kruskal-Wallis test with multiple comparison (A), the two-tailed Mann-Whitney test (B-D) or one-way ANOVA with multiple comparisons (E).

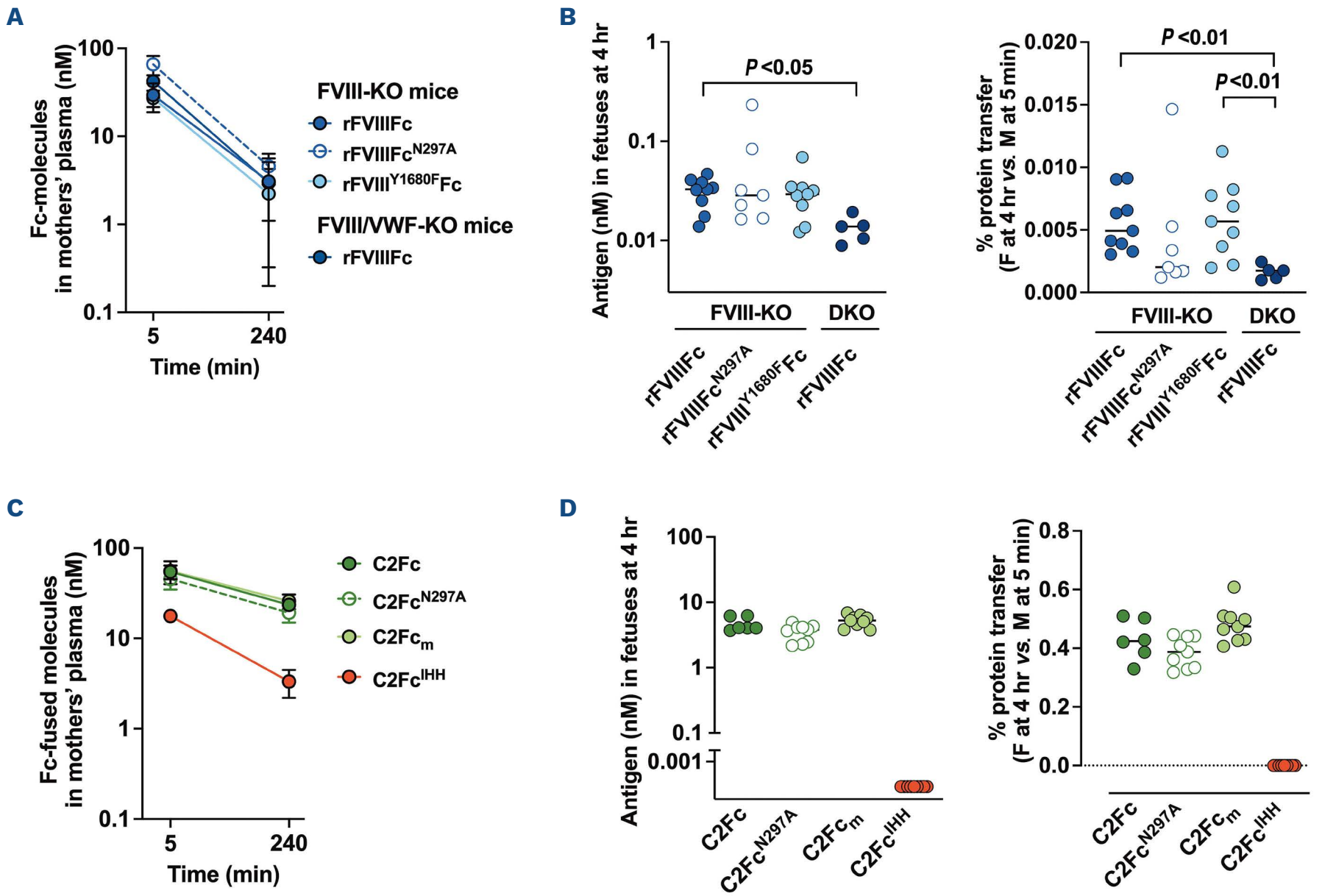

Figure 2. Low transplacental delivery of factor VIII fused to the Fc fragment of human IgG1 is not due to binding to FcγR or von Willebrand factor. (A-D) Naïve pregnant hemophilia A (HA) mice (N=3) were injected intravenously (iv) at day 17.5 of gestation with factor VIII (FVIII) fused to the Fc fragment of human IgG1 (rFVIIIFc) variants (225 pmoles in A and B) or C2Fc variants (170 pmoles in C and D). After 5 minutes (min) and 4 hours (hr), blood was collected from the mothers (M) to quantify the antigens by ELISA. Quantifications are depicted in nanomolar concentrations (mean±Standard Deviation in A and C). After 4 hr, plasma of fetuses (F) was obtained to quantify the antigens using FVIII (B) or C2-specific (D) ELISA. Quantifications are depicted in nanomolar concentrations (left) or as percentage of antigen detected in the fetuses' plasma at 4 hr *versus* that detected in mothers' plasma at 5 min (i.e., percentage of protein transfer, right). Each dot represents a pool of plasma from 2 to 3 fetuses. Horizontal lines depict the means. Differences were statistically assessed using the Kruskal-Wallis test with multiple comparison.

whether the transplacental delivery of rFVIIIFc impacts the capacity of the progeny to develop an anti-FVIII allo-immune response. Following replacement therapy using therapeutic FVIII, the offspring from pregnant mice injected with rFVIIIFc developed levels of anti-FVIII IgG (mean±SD: 14.1±17.7 μg/mL) and of FVIII inhibitors (6.7±7.2 BU/mL) similar to that developed by the progeny from mice injected with a control human monoclonal IgG (6.8±11.4 μg/mL and 3.3±6.6 BU/mL) (Figure 1C). In contrast, and in agreement with our earlier work performed with the FVIII A2 and C2 domains fused to the mouse Fcγ1,[10] the co-administration to pregnant HA mice of A2 and C2 fused to the human Fcγ1 was associated with a reduction in both the levels of anti-FVIII IgG (0.6±1.5 μg/mL) and FVIII neutralizing antibodies (0.6±1.3 BU/mL) in the offspring following treatment with BDD-FVIII (8.5±14.2 μg/mL and 2.2±3.2 BU/mL in control mice) (Figure 1D). Taken together, the data suggest that the human nature of the Fc fragment is not responsible for the lack of induction of tolerance to FVIII by rFVIIIFc in HA mice.

To obtain an insight into the potential reasons for the lack of induction of immune regulation upon administration of rFVIIIFc, we injected pregnant mice with rFVIIIFc, A2Fc, C2Fc, or rFIXFc and compared the amounts of the different proteins transferred to the fetuses' plasma. Of note, the four molecules presented comparable binding affinities for murine and human (*Online Supplementary Tables S1, S2*) FcRn at pH 6. Four hours after injection to the mothers, the concentration of rFVIIIFc in fetal plasma (0.03±0.01 nM) (Figure 1E, left) was 57-, 360-, and 5-fold lower than that of A2Fc (1.7±0.29 nM), C2Fc (10.8±1.83 nM), or rFIXFc (0.15±0.02 nM), respectively (Figure 1E, left). Accordingly, the percentage of transferred proteins calculated according to the protein

concentration measured in the plasma of the fetuses at 4 hr *versus* that measured in the plasma of the mothers at 5 min were close to 2-log lower for rFVIIIFc (0.005±0.002) as compared to A2Fc (0.38±0.06), C2Fc (0.56±0.25), and rFIXFc (0.12±0.02) (Figure 1E, right).

The transplacental delivery of factor VIII fused to the Fc fragment of human IgG1 is not affected by binding of its Fc fragment to FcγR or of its factor VIII moiety to von Willebrand factor

To investigate whether binding of the Fc moiety of rFVIIIFc to FcγR is involved in the poor transplacental delivery of rFVIIIFc, we used a rFVIIIFc^{N297A} mutant that does not bind to FcγR (*Online Supplementary Figure S1A*) but maintains unperturbed binding affinity for FcRn at pH 6 (*Online Supplementary Tables S1, S2*). The rFVIIIFc^{N297A} mutant presented the same rate of elimination after 4 hr of injection in pregnant HA mice, as the standard rFVIIIFc (Figure 2A). Moreover, rFVIIIFc^{N297A} crossed the placenta to the same extent as rFVIIIFc (Figure 2B). In agreement with this, the C2Fc^{N297A} variant did not bind to FcγR (*Online Supplementary Figure S1A*) and presented similar rates of elimination from the mothers' circulation (Figure 2C) and levels of transplacental delivery (Figure 2D) as C2Fc. Importantly, the C2 domain fused to the Fc fragment of murine IgG1 (C2Fc$_m$) demonstrated similar elimination rates (Figure 2C) and transplacental delivery as C2 fused the human IgG1 Fc (Figure 2D), indicating that the use of a xenogeneic Fc fragment did not alter the placental transfer of C2Fc in mice. As a control, we also generated a C2Fc^{IHH} mutant that does not bind to the FcRn at acidic pH (*Online Supplementary Figure S1C*). Introduction of the IHH mutations drastically increased the rate of elimination of C2Fc from the mothers' circulation (Figure 2C) and abrogated the transplacental delivery of the protein (Figure 2D), confirming that binding to FcRn is essential for the maintenance of the Fc-fused molecules in circulation and their transplacental passage.

Once in the circulation, rFVIIIFc binds to the chaperone protein of FVIII, vWF.[21] We investigated whether the binding of rFVIIIFc to vWF affects the transplacental delivery of the molecule due to the large size of the complex. We used the rFVIII^{Y1680F}Fc variant with impaired binding to vWF (*Online Supplementary Figure S1B*) and observed no modification in the rate of elimination from the mothers' circulation (Figure 2A) or in levels of protein crossing the placenta (Figure 2B). Likewise, injection of rFVIIIFc to HA mice and DKO mice yielded similar pharmacokinetics in the mothers' circulation (Figure 2A), although the transplacental delivery of rFVIIIFc was slightly lower in DKO mice than in HA mice (Figure 2B).

Factor VIII fused to the Fc fragment of human IgG1 is retained in the mothers' organs, and low levels of the protein reach and cross the placenta

To better understand the low levels of rFVIIIFc reaching the fetal circulation, we performed *in vivo* imaging in HA mice after the injection of rFVIIIFc or C2Fc or C2Fc^{IHH} conjugated with Alexa Fluor 680 at day 17.5 of gestation. A greater accumulation of rFVIIIFc in the liver, kidney and spleen of HA mice was observed when compared with C2Fc and C2Fc^{IHH} (Figure 3A). Conversely, low levels of rFVIIIFc reached the placenta and the amounts of rFVIIIFc that were transferred to the fetal circulation were not visible. High levels of C2Fc in the placenta and fetuses' side were observed, while C2Fc^{IHH} accumulated in, but did not cross, the placenta (Figure 3A).

Using the human placental cell line BeWo, we studied the levels of endocytosis of rFVIIIFc and compared them to that of C2Fc and C2Fc^{IHH}. Our results indicate that rFVIIIFc co-localized with early endosomes (EEA1), albeit to a lesser extent than C2Fc and C2Fc^{IHH} (Figure 3B), indicating reduced endocytosis. In addition, chasing experiments demonstrated that higher percentages of internalized rFVIIIFc and C2Fc^{IHH} were routed towards lysosomes (co-localized with dextran) than C2Fc (Figure 3C). Owing to the very low production yields of A2Fc as compared to C2Fc, similar experiments could not be performed with A2Fc and A2Fc^{IHH} as additional controls.

Contribution of protein size and positive electrostatic potential to the low transplacental delivery of factor VIII fused to the Fc fragment of human IgG1

Correlation of the size of the Fc-porter molecules with their percentage of protein transfer from the mothers' to the fetuses' blood, suggested that larger molecules tend to present poor transplacental deliveries, while smaller molecules cross the placental barrier better. Yet, some molecules with similar molecular weights, e.g., m66.6 and VRC01 (two human anti-HIV monoclonal IgG), presented slight differences in their capacity of being transferred from mothers to fetuses (*Online Supplementary Figure S3*). The presence of positively charged patches on the surface of the variable fragments (Fv) of IgG often results in faster blood clearance and increased tissue retention,[22] owing to the engagement of electrostatic interactions with the FcRn at neutral pH and hampered FcRn-mediated recycling. To determine whether the presence of positive charges in the Fv of IgG also alters FcRn-dependent transplacental delivery, we modeled the Fab fragments of m66.6 and VRC01, that share identical Fc fragments but have different Fv. Modeling of the Fab fragments predicted that m66.6 displays a drastically stronger positive electrostatic potential than VRC01 (Figure 4A). In agreement with our hypothesis, injection of m66.6 to pregnant HA mice resulted in a 1.9-fold lower transplacental delivery of the IgG than that achieved upon injection of VRC01 (Figure 4B). Calculation of the charge distribution on the BDD-FVIII structure highlighted the C1 and C2 domains, and a portion of the A3 domain as moieties with a high positive electrostatic potential that was somewhat reminiscent of the situation with m66.6 and may account for the poor transplacental delivery of the molecule. To test this hypothesis, we generated a rFVIII^{C1C2}Fc

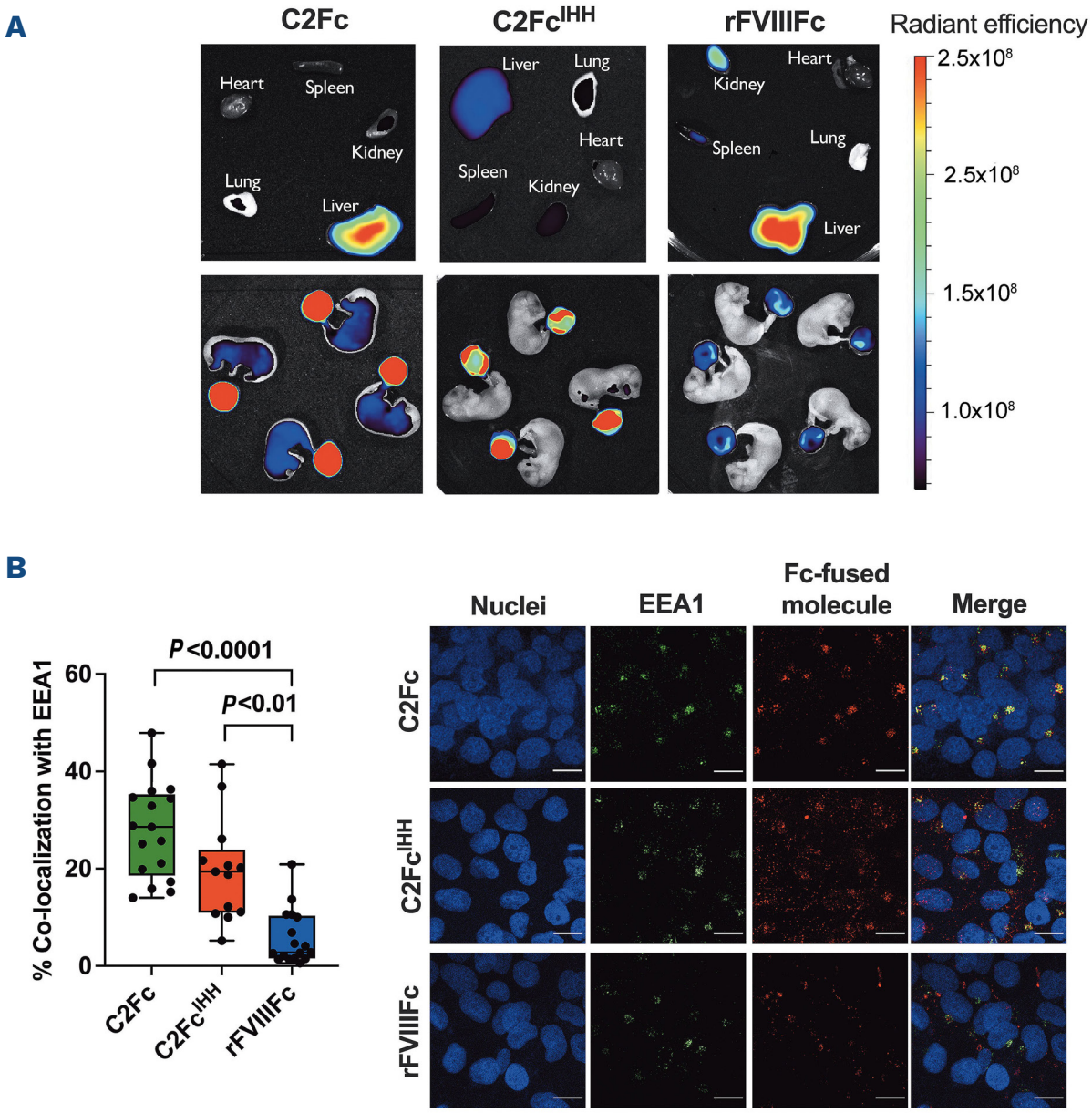

Figure 3. The distribution of factor VIII fused to the Fc fragment of human IgG1 differs from that of C2Fc. (A) Naïve pregnant hemophilia A (HA) mice were injected intravenously (iv) at day 17.5 of gestation with Alexa Fluor 680 labeled C2Fc (170 pmoles), C2Fc^{IHH} (170 pmoles) or factor VIII (FVIII) fused to the Fc fragment of human IgG1 (rFVIIIFc) (225 pmoles). After 4 hours (hr), the organs from the mothers as well as the fetuses and their placentas were collected to perform *in vivo* imaging. (B) Endocytosis of Fc-fused molecules by BeWo cells. Fc-fused molecules (114 nM) were incubated with 2x10^5 BeWo cells for 30 minutes (min). Immunofluorescence was measured (EEA1: green; Fc-fused molecules: red; nuclei: blue). The graph depicts the percentages of co-localization between the Fc-fused molecules and EEA1. (C) Lysosomal routing of Fc-fused molecules in BeWo cells. Fc-fused molecules (114 nM) were incubated with 2x10^5 BeWo cells for 30 min, the cells were then washed and incubated in medium alone for an additional 6 hr. Immunofluorescence was measured (dextran: green; Fc-fused molecules: red; nuclei: blue). The graph depicts the percentages of co-localization between the Fc-fused molecules and dextran. Experiments were performed at least 3 times in duplicates. Representative immunofluorescence images of EEA1 (B) or dextran (C) staining in BeWo cells are shown on the right. Scale bars represent 20 μm. Statistical analyses were performed using the Kruskal-Wallis test with multiple comparison (B) or one-way ANOVA with multiple comparisons (C).

variant wherein R2090, K2092, F2093, R2215 were mutated to alanines.[23,24] As a control, we generated a rFVIII^{N2118Q}Fc variant where the N2118Q mutation removes the negatively charged N-linked glycan in the C1 domain of the protein.[25] Modeling of the rFVIII^{C1C2} moiety of the variant predicted a decrease in the positive electrostatic potential of the C1 and C2 domains (Figure 4A). Interestingly, the percentage of protein transfer of the rFVIII^{C1C2}Fc variant (0.032±0.022 nM) was 8-fold greater than that of rFVIIIFc (0.004±0.003) (Figure 4C). In contrast, the removal of the N-linked glycan in the C1 domain of rFVIII^{N2118Q}Fc prevented the transplacental delivery of the molecule (Figure 4C). Importantly, all rFVIIIFc variants and antibodies demonstrated similar binding to FcRn (*Online Supplementary Tables S1, S2*) and vWF (*Online Supplementary Figure S1B*).

To further assess the respective contribution of individual FVIII domains to the low transplacental delivery of rFVIIIFc, we generated different FVIII domains fused to the human

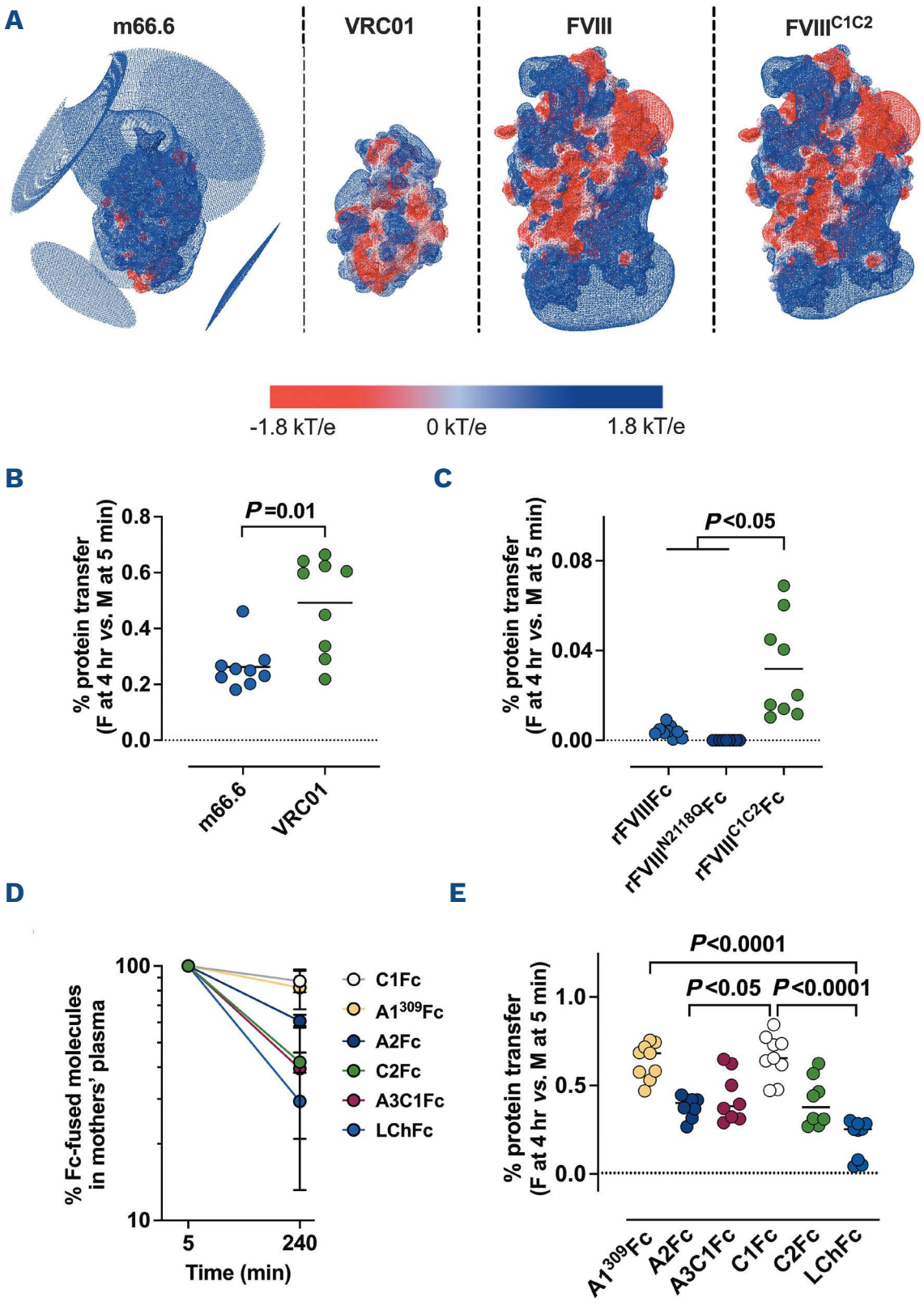

Figure 4. The presence of positively charged patches hampers the transplacental delivery of Fc-fused molecules. (A) The 3D structure of human anti-HIV m66.6 Fab (PDB: 4NRZ), human anti-HIV VRC01-class DRVIA7 Fab (PDB: 5CD5), BDD-FVIII (PDB: 6MF2), and the modeled FVIII^{C1C2} were used to calculate the surface electrostatic potentials with the Poisson-Boltzmann method (SWISS-PDB viewer). Negative potentials are depicted in red and positive potentials in blue. (B and C) Naïve pregnant hemophilia A (HA) mice (N=3) were injected intravenously (iv) at day 17.5 of gestation with IgG m66.6 or VRC01 (170 pmoles, in B) or rFVIIIFc variants (225 pmoles, in C). Four hours later, fetuses' plasma was collected to quantify the antigens by ELISA. The percentage of protein transfer are depicted as the percentage of antigen detected in the fetuses' plasma at 4 hours (hr) *versus* that detected in mothers' plasma at 5 minutes (min). (D and E) Naïve pregnant HA mice (N=3) were injected iv at day 17.5 of gestation with Fc-fused FVIII domains (170 pmoles). Five min and 4 hr later, blood was collected from the mothers to quantify the antigens by ELISA (mean±Standard Deviation, in D). After 4 hr, the antigens were quantified in the fetal circulation by ELISA. The graph depicts percentage of protein transfer (E). Each dot represents a pool of plasma of 2 to 3 fetuses' plasma, horizontal lines depict the means, the dotted line represents the percentage of protein transfer of rFVIIIFc, as calculated in Figure 1E. Differences were statistically assessed using the two-tailed Mann-Whitney test (B) or the Kruskal-Wallis test with multiple comparison (C and E).

Fcγ1 and evaluated their transplacental delivery. The LChFc presented the fastest clearance from the mothers' blood and the Fc-fused A1 and C1 domains presented the longest residence times (Figure 4D). Accordingly, the percentage of protein transfer of the LChFc was significantly lower than that of other Fc-fused domains, albeit 36-fold higher than rFVIIIFc (Figure 4E). In contrast, the A1Fc, A2Fc, A3C1Fc, C1Fc and C2Fc exhibited 114-, 68-, 104-, 118-, 73-fold higher percentage of protein transfer than rFVIIIFc, respectively (Figure 4E). We were not able to generate the A3Fc and C1C2Fc domains.

Exposure to factor VIII-derived immunodominant peptides during fetal life reduces the immune response towards factor VIII in hemophilia A mice

The co-injection of 1,500 pmoles of A2Fc and C2Fc (i.e., 20% of the FVIII protein) to pregnant HA mice was able to reduce the immune response to exogenous FVIII in the progeny (Figure 1D). Here, we investigated whether the injection of a pool of Fc-fused FVIII domains that covers the entire sequence of the molecule could completely prevent the onset of the anti-FVIII alloimmune response. The offspring from pregnant HA mice injected with pooled A1Fc, A2Fc, A3C1Fc, C1Fc and C2Fc (1,500 pmoles of each), developed levels of anti-FVIII IgG (3.1±4.0 μg/mL) and inhibitory antibodies (2.0±3.8 BU/mL) similar to the offspring from control mice (8.5±14.2 μg/mL and 2.2±3.2 BU/mL) (Figure 5A, B).

We then explored whether the transfer of Fc-fused immunodominant FVIII peptides could resume immune protection against exogenous FVIII. We designed two Fc-fused

molecules carrying three and four FVIII-derived immunodominant peptides as identified by Steinitz *et al.*:[26] iPep-FVIIIFc 1 included peptides from the A3, A2 and A1 domains, iPep-FVIIIFc 2 included a different peptide from the A1 domain, 2 C1 peptides and one C2 peptide (Figure 5C, *Online Supplementary Table S3*). The peptides were separated by different XTEN blocks[27] (*Online Supplementary Table S4*). The iPep-FVIIIFc 1 and 2 exhibited 120- and 254-fold higher percentage of protein transfer than rFVIIIFc, respectively (Figure 5D). Injection of pregnant HA mice with 1,500 pmoles of each construct led to a significant decrease in levels of anti-FVIII IgG (1.3±3.5 μg/mL) (Figure 5A) and FVIII neutralizing antibodies (0.9±2.6 BU/mL) (Figure 5B) in the progeny following 5 injections of BDD-FVIII, in comparison to the offspring from control mice treated with IgG1. Statistical significance was already observed after 4 injections (*P*=0.0012) (*Online Supplementary Figure S4*) and was maintained until the 6th injection (*P*=0.0041).

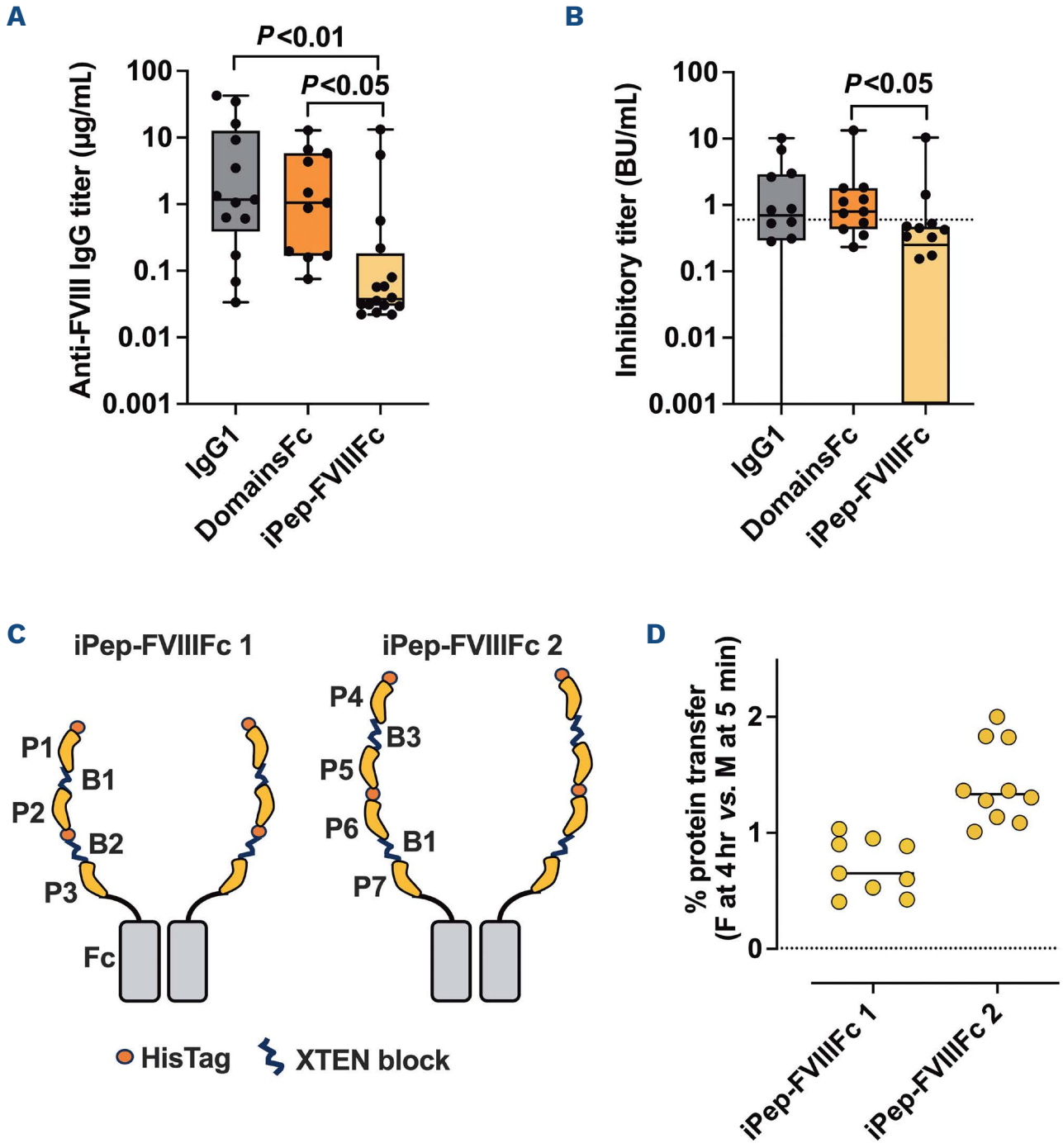

Figure 5. Reduction of the immune response towards factor VIII in progeny from mothers injected with Fc-fused factor VIII-derived immunodominant peptides during pregnancy. (A and B) The humanized monoclonal IgG1, trastuzumab (1,500 pmoles, Herceptin®), a pool of Fc-fused factor VIII (FVIII) domains (A1Fc, A2Fc, A3C1Fc, C1Fc and C2Fc, 1,500 pmoles each) or a pool of iPep-FVIIIFc 1 and 2 (1,500 pmoles each) were injected intravenously (iv) into pregnant hemophilia A (HA) mice at E16, E17 and E18. The 4-week-old progeny was injected weekly with BDD-FVIII (1 μg/mouse). After 5 injections, blood was collected, anti-FVIII IgG titers were quantified by ELISA (expressed in μg/mL, in A), and inhibitory titers were quantified by chromogenic FVIII assay (dotted line: limit of detection of inhibitory IgG, i.e., 0.6 BU/mL) (B). Each dot represents one mouse. (C) Schematic depiction of the iPep-FVIIIFc molecules (see *Online Supplementary Tables S3* and *S4* for details). (D) Naïve pregnant HA mice (N=3) were injected iv at day 17.5 of gestation with 170 pmoles of iPep-FVIIIFc 1 or 2. After 4 hr, the antigens were quantified in the fetal circulation by ELISA. The graph depicts percentage of protein transfer. Each dot represents a pool of plasma of 2 to 3 fetuses' plasma. Horizontal lines depict the means. The dotted line represents the mean of percentage of protein transfer of rFVIIIFc, as calculated in Figure 1E. Differences were statistically assessed using the Kruskal-Wallis test with multiple comparison.

Discussion

We have demonstrated in the past that the transplacental delivery of the A2 and C2 domains of FVIII fused to the Fc fragment of the mouse IgG1 drastically reduces the anti-FVIII immune response to exogenous FVIII following replacement therapy in the offspring from HA mice. Reduction in levels of anti-FVIII IgG and of neutralizing antibodies was associated with the induction of FVIII-specific Tregs. However, the progeny of the treated mothers still developed clinically relevant levels of FVIII neutralizing antibodies.[10] Here, we investigated whether the transplacental delivery of the complete FVIII molecule may induce complete immune tolerance towards therapeutic FVIII. To this end, we first evaluated whether Fc-fused FVIII injected intravenously to pregnant HA mice is delivered to the circulation of the fetuses. Injection of rFVIIIFc to pregnant HA mice resulted in transcytosis of the molecule to the fetuses' blood. Transcytosis was time-, dose- and FcRn-dependent. Four hours after injection to the mothers, the circulating concentration of FVIII antigen in fetuses' plasma was 0.03±0.01 nM. Such an amount represents 2.5% of the normal FVIII plasma levels in the human (i.e., 1.2±0.6 nM[28]) and is in line with our earlier results wherein FVIII in the maternal blood was delivered from the circulation of the mothers to that of the fetuses using monovalent anti-FVIII IgG (FabFc),[29] and recent results obtained with rFIXFc in hemophilia B mice.[30] Importantly, FVIII retained its pro-coagulant potential along the transcytosis process. The prenatal delivery of rFVIIIFc, however, failed to prevent the immune response to therapeutic FVIII in the progeny, in contrast to the transplacental delivery of the pooled A2Fc and C2Fc molecules. Of note, HA mice were used in the present work, which represents a major difference with the typical clinical situation where carrier mothers have a normal *F8* allele and produce functional FVIII.

Comparison of the transplacental delivery of rFVIIIFc with that of other Fc-fused molecules highlighted the low potency for transcytosis of FVIII. Indeed, the percentage of protein transfer of rFVIIIFc was 5-fold lower than that of rFIXFc, 36-fold lower than that of the FVIII light chain, and from 57- to 118-fold lower than that of the individual Fc-fused FVIII domains. We tested several hypotheses to explain the poor transplacental delivery of rFVIIIFc. Among these, we have previously shown[29] that binding to endogenous vWF or to FcγR expressed on the mother's cells played little, if any, role, as confirmed here using rFVIII^{Y1680F}Fc and FVIIIFc^{N297A} variants that do not bind vWF or FcγR, respectively, or DKO mice. Our earlier work had identified the size of the molecules as an important limitation to efficient transplacental delivery. Thus, complexes were better transplacentally delivered when an anti-FVIII FabFc was associated with the FVIII C2 domain (20 kDa), than with BDD-FVIII (170 kDa), and the lowest transcytosis levels were achieved with full-length FVIII (280 kDa).[29] In the present study, we also observed that larger molecules (rFVIIIFc, 220 kDa) cross the placenta at lower levels than medium-sized molecules (rFIXFc, 120 kDa, or FVIII LChFc, 130 kDa), with individual Fc-fused FVIII domains and Fc-fused FVIII peptides crossing at the highest levels. However, VRC01 (150 kDa) crossed the placental barrier more efficiently than rFIXFc or FVIII LCh-Fc, suggesting that biophysical properties other than the mere size of the molecules also influence the efficiency of transplacental delivery.

A role for residues in the FVIII LCh in the poor transplacental delivery of FVIII had been initially suggested by our work using monovalent anti-FVIII IgG: FabFc specific for the FVIII C2 domain (derived from BO2C11)[31] or C1 domain (derived from KM33)[32] were able to transfer FVIII from the blood of pregnant mice to that of the fetuses, while a FabFc specific for the FVIII A2 domain failed to do so.[29] The latter observations are substantiated by our present finding that the LChFc was less efficiently transcytosed than individual Fc-fused FVIII domains. In addition, the Fc-fused A3C1 and C2 domains were delivered to similar extents, but at lower levels than the Fc-fused C1 domain. Taken together, the results suggest a synergy between residues in the A3 and C2 domains in reducing the transcytosis of FVIII LCh. The hypothesis could not be tested owing to our failure to produce the individual A3Fc and C1C2Fc fusion molecules. Of note, roles for the FVIII C1 and C2 domains had been documented in FVIII binding to platelets[33] as well as in FVIII endocytosis by human dendritic cells and circulating half-life in mice.[23]

In the case of IgG, the presence of positively charged patches in the IgG Fv fragment is associated with a higher tissue accumulation.[22] Furthermore, IgG with positively charged Fv domains binds FcRn at neutral pH, which impairs their FcRn-mediated recycling, and affects their pharmacokinetics.[34,35] In line with this, we show here that m66.6, a monoclonal IgG containing an Fv with a strong positive electrostatic potential, was less efficiently transferred from the circulation of the mothers to that of the fetuses than VRC01, an IgG with an identical Fc but with a less positively charged Fv fragment. Our results thus suggest that the presence of a strong positive electrostatic potential in the variable regions of antibodies not only affects their pharmacokinetics, but also their transplacental delivery.

Interestingly, the epitopes targeted by KM33 and BO2C11 on the FVIII C1 and C2 domains contain positively charged residues.[24,36] The FVIII LCh is thus endowed with a strong positive electrostatic potential, which is absent from the FVIII heavy chain, or from the light chain of factor V (*Online Supplementary Figure S5*). Removal by site directed mutagenesis of three positively charged residues in the FVIII C1 and C2 domains of rFVIIIFc drastically reduced the positive potential of its LCh, and was associated with a 5.7-fold increase in transplacental delivery. Whether

mutation of additional charged residues in the FVIII A3 domain may further foster the transplacental delivery of rFVIIIIFc needs to be investigated. Interestingly, a novel therapeutic rFVIIIFc molecule, efanesoctocog alfa (ALTUVIIIO™, Sanofi-SOBI) has recently reached the clinic for the prevention of bleeds in HA patients.[37] In efanesoctocog alfa, rFVIIIFc is associated with the Fc-fused D'D3 domains of vWF and with two large XTEN insertions of 144 and 288 amino-acids.[38] Efanesoctocog alfa demonstrated a 2-fold increased half-life in comparison with standard rFVIIIFc.[39] Future work should explore whether shielding the C1, C2 and part of the A3 domains of FVIII by D'D3 and/or the XTEN insertions[40] potentiates transplacental delivery, notwithstanding the significant increase in molecular weight and hydrodynamic radius of the molecule. Injection of pregnant HA mice with A2Fc and C2Fc reduced the FVIII-specific humoral immune response in the offspring following FVIII replacement therapy. Unexpectedly, however, injection of the pooled available Fc-fused FVIII domains (A1Fc, A2Fc, A3C1Fc, C1Fc and C2Fc) failed to protect the progeny of the treated mothers from the development of neutralizing anti-FVIII IgG. The FVIII A2 and C2 domains carry most of the epitopes recognized by inhibitory anti-FVIII antibodies in humans.[41–44] In contrast, the immunodominant T-cell epitopes of human FVIII determined in HA mice are more frequent on the A1 domain than on the A2, C1, A3 or C2 domains.[26] Epitope density is a key parameter for efficient immune responses: antigens with higher concentrations of MHC-binding motifs per amino acid tend to be better recognized by the immune system.[45,46] 'Immuno-focusing' has been developed as a strategy to potentiate vaccine efficacy using epitope-enriched immunogens derived from viruses.[47] Whether the same phenomenon applies to the induction of immune tolerance is not known.

There is a possibility that our simultaneous use of all FVIII domains resulted in a 'dilution' of those epitopes that are the most important for tolerance induction. We thus designed an alternative strategy wherein we produced two Fc-fused molecules that contain three and four of the immunodominant $CD4^+$ T-cell FVIII epitopes identified by Steinitz *et al*. in hemophilic E17 C57Bl6 mice, respectively.[26] Some of the peptides are also immunodominant T-cell epitopes in healthy humans.[48] The co-injection of both constructs to pregnant HA mice led to their transplacental delivery and was associated with a reduction in the FVIII-specific immune response in the offspring following weekly replacement therapy with BDD-FVIII. Future work should determine whether the transplacental delivery of immunodominant peptide(s)-Fc fusion proteins is a suitable strategy to reprogram immune repertoires during fetal life and confer specific and long-lasting immune tolerance in different physio-pathological contexts, such as alloimmune responses to drugs, autoimmunity, and allergy.

Disclosures

SLD is co-inventor in two patents related to Fc-fused proteins (US20220175896A1 and US20170072032A1). None of the other authors have any conflicts of interest to disclose.

Contributions

ARR, SD, MTG, DL, JDD and SLD designed the research. ARR, SD, AA, VD, LD, AM, GMC and MTG performed the experiments. ARR, SD, AA, MTG, DL, JDD and SLD analyzed the results. ARR, SD and SLD wrote the manuscript.

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Data-sharing statement

All data are included in the paper or in the Online Supplementary Appendix. Additional data will be shared on reasonable request to the corresponding author.

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