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Recombinant factor VIII Fc (rFVIIIFc) fusion protein reduces immunogenicity and induces tolerance in hemophilia A mice

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Abstract

Anti-factor VIII (FVIII) antibodies is a major complication of FVIII replacement therapy for hemophilia A. We investigated the immune response to recombinant human factor VIII Fc (rFVIIIFc) in comparison to BDD-rFVIII and full-length rFVIII (FL-rFVIII) in hemophilia A mice. Repeated administration of therapeutically relevant doses of rFVIIIFc in these mice resulted in significantly lower antibody responses to rFVIII compared to BDD-rFVIII and FL-rFVIII and reduced antibody production upon subsequent challenge with high doses of rFVIIIFc. The induction of a tolerogenic response by rFVIIIFc was associated with higher percentage of regulatory T-cells, a lower percentage of pro-inflammatory splenic T-cells, and up-regulation of tolerogenic cytokines and markers. Disruption of Fc interactions with either FcRn or Fcγ receptors diminished tolerance induction, suggesting the involvement of these pathways. These results indicate that rFVIIIFc reduces immunogenicity and imparts tolerance to rFVIII demonstrating that recombinant therapeutic proteins may be modified to influence immunogenicity and facilitate tolerance.

Keywords

Hemophilia A; Immune tolerance; Regulatory T cells; FcRn; Fc fusion protein; Immunogenicity; Factor VIII

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Author contributions

S.K, H.J, and T.L, designed the study and performed data analysis, S.K and H.J wrote the manuscript. S.K, T.L, D.D, S.P-W, and E.S-C performed study, data, and statistical analyses; R.P, G.F.P, D.L, R.S.B, and N.J contributed to critical evaluation of the work and assisted in manuscript preparation.

Conflict of interest

S.K, T.L, D.D, S.P-W, E.S-C, and R.P are Biogen employees and own equity in the company. G.F.P and H.J own equity in and were former employees of Biogen. R.S.B, D.L, and N.J are consultants for Biogen.

1. Introduction

Hemophilia A is an X-linked inherited bleeding disorder characterized by spontaneous and traumatic bleeding [1]. The pathophysiologic features of this disease are associated with very low levels or activity of factor VIII (FVIII) protein, arising because of genetic defects (e.g. intron 22 inversion, large deletions) [2]. Currently, the mainstay of treatment for hemophilia A is protein replacement therapy [3], one major complication of which is development of neutralizing antibodies, also known as inhibitors, to the infused FVIII. The incidence of inhibitor formation is estimated at 20–30% in all patients and at 30–40% in patients with severe disease.[4] The development of inhibitors results from a complex multifaceted immune response involving both genetic and environmental risk factors [5,6]. Several key molecules have been identified that correlate with inhibitor formation in patients with hemophilia. These include polymorphisms in the genes of the proinflammatory cytokine tumor necrosis factor-α (TNF-α), the anti-inflammatory cytokine interleukin-10 (IL-10), and the regulatory T cell (Treg) marker cytotoxic T-lymphocyte antigen-4 (CTLA-4). Higher levels of TNF-α and IL-10 have been demonstrated to correlate with higher incidence of inhibitors while higher CTLA-4 expression has been associated with a decreased incidence of inhibitors [7–9]. However, the presence of splenic IL-10 positive T-cells has also been associated with induction of FVIII tolerance in Hem A mice [10,11].

Interventions to mitigate rFVIII immunogenicity in experimental models have included impairing co-stimulatory signals during antigen presentation [12], inducing Tregs [13], presentation of FVIII antigen by immature dendritic cells [14], and designing FVIII molecules with fewer putative immunogenic epitopes. We therefore sought to investigate the immunogenicity and immune tolerance potential of recombinant FVIII Fc fusion protein (rFVIIIFc), which was recently approved as a long-acting FVIII replacement therapy for patients with hemophilia A. rFVIIIFc is composed of a single molecule of B-domain deleted factor VIII fused to the Fc domain of human IgG1 [15,16]. The Fc portion enables the molecule to interact with the neonatal Fc receptor (FcRn), replicating the interaction that rescues IgG from lysosomal degradation pathways, resulting in a prolonged circulating half-life [17]. Immunomodulatory properties of Fc-containing fusion proteins have also been reported previously [18]. Of interest, two T-cell epitopes, termed Tregitopes, have been identified in the Fc region of IgG1 that are capable of activating Tregs [19,20].

In this report, we evaluated antibody and cellular immune responses to rFVIIIFc in hemophilia A mice and interrogated the pathways that potentially mediate rFVIIIFc immune tolerance. We also investigated receptor dependent mechanisms to delineate the possible downstream molecules that may promote the tolerogenic activity of rFVIIIFc.

2. Materials and methods

2.1. Mice

Hemophilia A (HemA) mice (C57BL/6) bearing a FVIII exon 16 knockout on a 129 × B6 background [21] were obtained from Dr. H. Kazazian (University of Pennsylvania). All animal procedures used were approved by the Institutional Animal Care and Use Committee and performed based on guidelines from the Guide to the Care and Use of Laboratory Animals.

2.2. Antibodies and reagents

Antibodies for FACS were obtained from BD Biosciences (Franklin Lakes, NJ) or eBioscience (San Diego, CA). Recombinant human B-domain-deleted FVIIIFc (rFVIIIFc), recombinant human B-domain-deleted FVIII (Biogen in-house produced) used in ELISA, rFVIIIFc IHH (amino acid substitutions I253A, H310A, H435A) and rFVIIIFc N297A (single amino acid substitution in the Fc domain) were produced as previously described [16]. Recombinant factor VIII products BDD-rFVIII Xyntha® (Wyeth Pharmaceuticals, Philadelphia, PA) and full-length FVIII Advate® (Baxter Healthcare Corporation, Westlake Village, CA) were purchased and reconstituted according to manufacturers' instructions.

2.3. Immunization/tolerance induction in mice

The study scheme for immunization and/or tolerance induction is depicted in Fig. 1A and B. Three treatment groups consisting of 8–10 week old male HemA mice received intravenous doses of 50, 100, or 250 IU/kg on days 0, 7, 14, 21, 35, and 53. Blood samples were collected by retro-orbital bleeding prior to dosing on days 0, 14, 21, 28 and 42. Plasmas were prepared, and anti-BDD-FVIII total binding and neutralizing antibody levels were determined using ELISA and Bethesda assay, respectively. Animals were euthanized on day 56 by CO_2 inhalation and spleens were dissected in sterile PBS to isolate single cell suspensions (Miltenyi Biotec, Cologne, Germany) and were either fixed in 3% formalin for FACS staining or stored in dissociation buffer for RNA isolation (Roche Applied Science, Indianapolis, IN). For immune tolerance studies, mice were first injected with 50 IU/kg on days 0, 7, 14, 21, and 35, followed by 250 IU/kg of rFVIIIFc once weekly for 4 weeks. Rechallenged animals were tested for anti-BDD-FVIII antibody levels in plasma collected on days 14, 21, and 28 post rechallenge. To test the immune response to non-specific antigens, mice were injected subcutaneously on days 42 and 49 with DNP-OVA at 100 μg per mouse in a 1:1 emulsion with Titermax Gold adjuvant from Sigma®. Antibody responses to DNP and OVA were measured using an anti-DNP Ig and anti-OVA Ig assay kit from Assay Diagnostics.

2.4. Anti-BDD-FVIII antibody ELISA

The standard used for mouse IgG was a polyclonal pool of anti-FVIII monoclonal antibodies prepared by mixing equal amount of GMA8002 (A1), GMA8008 (C2), GMA8011 (C1), GMA8015 (A2), GMA8016 (A2), GMA8005 (A1/A3) (Green Mountain Antibodies Inc, Burlington, VT; FVIII domain epitopes in parenthesis). Detection antibody used was goat anti-mouse IgG-HRP. Absorbance was measured on a Spectramax M2 plate reader (Molecular Devices).

2.5. Bethesda assay for determining neutralizing antibody titers

Plasma samples were mixed with known concentrations of BDD-rFVIII (in-house prepared) and incubated for 2 h at 37 °C. Residual FVIII activity in the mixture was then tested using a

Coatest FVIII SP kit. The activity of FVIII was calculated against a standard curve generated with serially diluted BDD-rFVIII in naïve HemA mouse plasma.

2.6. FACS analysis

Splenic lymphocytes and dendritic cells were stained for surface and intracellular targets. For intracellular staining, cells were permeabilized with BD Fix-Perm solution (BD Biosciences) followed by incubation with respective antibodies in the same buffer. Fluorescence intensity was recorded using a BD FACS Canto II and analysis performed using FLOWJO software. For each sample 10,000 events were acquired on the flow cytometer. T-cells and dendritic cells were gated based on CD4+ and CD11c+ staining, respectively.

2.7. Real time PCR and real time PCR-based array analysis

Total RNA was isolated (Roche Applied Science, Indianapolis, IN) and reverse transcribed to cDNA (Qiagen, Hilden, Germany). PCR primers for the tested genes were designed and purchased from IDT technologies (Coralville, IA). SYBR green–based real-time PCR was carried out using Quantitect system (Qiagen, Hilden, Germany) or a PCR-based array for tolerance specific genes (PAMM047Z, T-cell Anergy and Immune Tolerance PCR Array; SA Biosciences, Frederick, MD) in an ABI 7900 Fast Block real-time PCR machine (Applied Biosystems, Foster City, CA). Results were analyzed using the 7500 software version 2.0.5 using the $2^{-\Delta Ct}$ relative quantification method [22], after normalization to GAPDH, HPRT, Hsp90ab, beta-actin, and GusB. mRNAs that displayed threshold cycles (Ct) >35 were excluded from the analysis.

2.8. T-cell proliferation and determination of interferon-γ (IFN-γ) levels

HemA mice (8–10 week old) were injected with rFVIII products once a week for 2 weeks. Seventy-two hours post the second injection mice were euthanized by CO_2 inhalation and splenic T-cells isolated using magnetic bead-based murine CD4+ T-cell isolation kit (Miltenyi Biotec, Germany). T-cells were then labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, Carlsbad, CA). Peritoneal macrophages were obtained from naïve HemA mice (8–10 weeks old) by euthanasia and peritoneal lavage with sterile PBS. Labeled T-cells from immunized mice were co-incubated with naïve peritoneal macrophages in the presence of BDD-rFVIII or vehicle or CD3/CD28 microbeads (positive control; Miltenyi Biotec) in X-VIVO 15 medium (Lonza) containing co-stimulatory antibodies namely anti-CD28 and anti-CD49d (BD Biosciences), for 96 h at 37 °C. IFNγ levels in the culture supernatant were measured using an ELISA kit from Meso Scale Devices (MSD). T-cell proliferation was determined by measuring CFSE fluorescence intensity (MFI) using FACS (BD FACS CANTO II).

2.9. Treg mediated suppression of in vitro effector T-cell interferon-γ secretion

T-effector cells were isolated from HemA mice injected twice with 250 IU/kg rFVIIIFc as described above. Tregs were isolated from HemA mice injected with 5 weekly injections of 50 IU/kg rFVIIIFc, using the murine CD4CD25 cell isolation kit (Miltenyi Biotec, Germany). Antigen presenting $CD90.2^{-}$ cells were isolated from naïve HemA mouse spleen using a magnetic bead based system (Miltenyi Biotec, Germany). The cells were reconstituted at various densities *in vitro* and activated with 10 nM of rFVIII in X-VIVO 15 medium (Lonza) containing co-stimulatory antibodies namely anti-CD28 and anti-CD49d (BD Biosciences), for 96 h at 37 °C. IFNγ levels in the culture supernatant were measured using an ELISA kit from Meso Scale Devices (MSD).

2.10. Statistical analysis

Statistical analyses of results were carried out either using unpaired 2-tailed student's *T*-test or Mann–Whitney's *T*-test. *p*-values < 0.05 was considered to be significant.

3. Results

3.1. rFVIIIFc evokes minimal antibody response at therapeutically relevant doses and induces FVIII-specific tolerance

To evaluate the antibody responses to rFVIIIFc in comparison with either BDD-rFVIII or full length rFVIII (FL-rFVIII), hemophilia A (HemA) mice (8–10 weeks old) were treated with repeated intravenous administration of each drug at 50, 100, or 250 IU/kg (Fig. 1A). Total binding antibodies (IgG) and neutralizing titers against BDD-FVIII were determined weekly after day 14 (Fig. 1A and B). No antibodies were detected in the vehicle only treated group (data not shown). The total anti-BDD-FVIII antibody response was significantly lower in animals that received 50 IU/kg and 100 IU/kg of rFVIIIFc compared to the same doses of BDD-rFVIII and FL-rFVIII (Fig. 1C). The numbers of mice with detectable anti-BDD-FVIII antibodies were 2 of 13 in the 50 IU/kg rFVIIIFc group and 3 out of 10 in the 100 IU/kg rFVIIIFc group. In comparison, anti-BDD-rFVIII antibodies developed in 8 out of 13 mice in the 50 IU/kg BDD-rFVIII group and 8 of 10 mice in the 100 IU/kg BDD-rFVIII group, and in 8 and 9 out of 10 mice in the 50 IU/kg and 100 IU/kg FL-rFVIII groups, respectively. At 250 IU/kg, all three treatments evoked high total anti-BDD-rFVIII antibody responses, which were not significantly different among the three FVIII proteins (Fig. 1C). Neutralizing antibodies to BDD-FVIII were detected in none of 13 mice from the 50 IU/kg rFVIIIFc group and 1 of 10 mice from the 100 IU/kg rFVIIIFc group (Fig. 1D). In comparison, a higher proportion of FL-rFVIII-injected animals (8 of 10) had detectable neutralizing antibodies against BDD-FVIII at 50 IU/kg, whereas 2 out of 13 mice injected with BDD-rFVIII had detectable neutralizing antibody levels at this dose level. At 100 IU/kg, both FL-rFVIII and BDD-rFVIII treated mice had a larger proportion of animals with neutralizing antibodies against BDD-FVIII compared to that observed with rFVIIIFc. At 250 IU/kg, all FVIII treatments elicited high titers of neutralizing antibodies (Fig. 1D). These results indicate that in HemA mice, rFVIIIFc at 50 and 100 IU/kg is less immunogenic and results in less inhibitor formation compared to FL-rFVIII and BDD-rFVIII.

To assess whether therapeutically relevant doses of rFVIIIFc could induce immune tolerance to FVIII, HemA mice were pretreated with 5 weekly injections of vehicle or 50 IU/kg rFVIIIFc, followed by challenge with 250 IU/kg of rFVIIIFc. Mice pretreated with 50 IU/kg of rFVIIIFc mounted a significantly lower BDD-FVIII antibody response compared to animals pretreated with vehicle (Fig. 1E). Notably, only 2 of 8 mice pretreated with 50 IU/kg rFVIIIFc developed antibodies. In comparison, all the vehicle pretreated mice developed antibodies. rFVIIIFc pretreated mice also had lower levels of neutralizing antibodies compared to vehicle pretreated mice (Fig. 1F), indicating that repeat dosing of rFVIIIFc at 50 IU/kg induced at least partial immune tolerance to FVIII in HemA mice. The tolerance was specific for FVIII, since rFVIIIFc pretreated mice mounted a robust antibody response to the antigens DNP and OVA, confirming that the mice did not develop general immunosuppression following rFVIIIFc treatment (Fig. 1G and H).

3.2. Mechanisms of cellular tolerance in rFVIIIFc-treated HemA mice

To understand the lower immunogenicity and tolerogenic effects of rFVIIIFc, we profiled splenic T-cells in rFVIIIFc-treated mice. rFVIIIFc at 100 IU/kg induced a significantly higher percentage of CD4+CD25+Foxp3+ T cells consistent with Treg cells compared to vehicle treated mice. Both BDD-rFVIII and FL-rFVIII treatments (Fig. 2A) were comparable to each other and not significantly different from the vehicle control. In addition, rFVIIIFc treatment also resulted in higher percentages of splenic CD4+ T-cells expressing CD279 (PD-1) (Fig. 2B), a recognized tolerogenic molecule [23]. Consistent with this, splenocytes from rFVIIIFc-treated mice also had significantly lower percentages of CD4+ T-cells positive for intracellular TNF-α compared to that observed in BDD-rFVIIIFc or FL-RFVIII-treated mice (Fig. 2C). Further, we also found that rFVIIIFc-treated mice exhibited higher percentages of CD11c+CD274+ (PD-L1) dendritic cells (Fig. 2D). Taken together, rFVIIIFc up-regulates both phenotypic Tregs and molecules associated with the immunosuppressive PD-L1:PD-1 pathway, which potentially reduces immunogenicity and induces tolerance to FVIII.

Suppression of the immune response to rFVIII was further demonstrated by the lack of recall response of splenic T-cells from rFVIIIFc-treated mice to rFVIII presented *in vitro*. T-cells from HemA mice treated with 50 IU/kg of rFVIIIFc did not show significant proliferation *ex-vivo* in the presence of rFVIII compared to that observed with T cells from control treated mice (Fig. 2E), with no induction of IFN-γ secretion (Fig. 2F). In contrast, T-cells from the 250 IU/kg rFVIIIFc treatment group showed a robust dose-dependent increase in proliferation (Fig. 2E) and secretion of IFN-γ in response to rFVIII exposure *ex-vivo* (Fig. 2F). In addition, Tregs isolated from mice treated with 5 weekly doses of 50 IU/kg rFVIIIFc, was able to suppress IFNγ production from effector CD4 + T-cells isolated from mice receiving two weekly doses of 250 IU/kg rFVIIIFc (Fig. 2G). This suggests the existence of Treg cells in spleen of mice receiving 50 IU/kg of rFVIIIFc that may participate in the suppression of T-cell responses to rFVIII. In summary, these results from *ex-vivo* studies support the observations from the splenic leukocyte profiling and suggest that rFVIIIFc treatment resulted in suppression of T-cell responses to rFVIII.

3.3. rFVIIIFc activates multiple molecular determinants in promoting tolerance

To identify the major pathways involved in the tolerance induced by rFVIIIFc, we performed transcriptional profiling of splenocytes from mice treated with vehicle, 50 IU/kg rFVIIIFc and 250 IU/kg rFVIIIFc, the latter being a dose which was not associated with functional evidence of tolerance (Fig. 3A). The results demonstrated the induction of several genes that are known to be involved in multiple pathways of tolerance and anergy in mice treated with 50 IU/kg rFVIIIFc (Fig. 3B). Results were validated with qPCR. In addition to the tolerance specific genes such as Foxp3, CTLA-4, and IL-10 (Fig. 3C–E), anergy associated genes such as Egr2, Dgka, and CBL-B (Fig. 3F–H), prostaglandin synthase 2 (PTGS2) and prostaglandin E2 receptor (PTGER2) (Fig. 3B) were all up-regulated in the splenocytes from mice treated with 50 IU/kg rFVIIIFc compared to vehicle and 250 IU/kg rFVIIIFc treated mice. Conversely, pro-inflammatory molecules such as CCL3 and STAT3 (Fig. 3B) were down-regulated in the 50 IU/kg rFVIIIFc group. Additional qPCR analysis also revealed up-regulation of TGF-β (Fig. 3I). The up-regulation of tolerogenic molecules such as IL-10, TGF-β, IL-35 and IDO-1 (Suppl.), and down-regulation of pro-inflammatory cytokines such as IL-17 (Suppl.) is consistent with the induction of a tolerogenic microenvironment in response to 50 IU/kg rFVIIIFc that is conducive to the suppression of antibody responses to rFVIII.

3.4. Role of FcRn and Fcγ receptors in rFVIIIFc-mediated immune tolerance

Because of the presence of the Fc moiety, the gain of immune tolerance function of rFVIIIFc may be attributed to the interaction of rFVIIIFc with either FcRn or Fcγ receptors, some of which are associated with immunosuppression (namely the Fcγ RIIb receptor) (Fig. 4A). To dissect the receptor-mediated effect of rFVIIIFc, we constructed two mutants – rFVIIIFc-N297A and rFVIIIFc-IHH (I253A, H310A, H435A), which abrogate Fc binding to the Fcγ and FcRn receptors, respectively [24,25]. rFVIIIFc N297A exhibited a comparable pharmacokinetic profile to that of rFVIIIFc in HemA mice, whereas the circulating half-life of rFVIIIFc-IHH was reduced relative to that of rFVIIIFc as expected, owing to the lack of recycling via FcRn when these amino acids were mutated (data not shown). Interestingly, neither mutant diminished the tolerogenic effects of rFVIIIFc following repeated dosing of 50 IU/kg in HemA mice. Thus, blocking either FcRn or FcRγ interaction does not abrogate the immune tolerance properties of rFVIIIFc at this therapeutic dose level in comparison to the consequences of lacking Fc entirely as observed with BDD-rFVIII and FL-rFVIII which did result in substantial antibody development at 50 IU/kg (Fig. 1C). In contrast, blocking FcγR interactions (rFVIIIFc N297A), and to a lesser extent blocking FcRn interactions (rFVIIIFc-IHH), attenuated the antibody response in the high dose (250 IU/kg) treatment group (Fig. 4B).

Although rFVIIIFc N297A and rFVIIIFc-IHH demonstrated immunogenicity at 50 IU/kg that was comparable to the wild-type rFIVIIIFc, both mutants, did exhibit a diminished induction of CD25+/Foxp3+ T cells consistent with Tregs and lower percentages of CD4+ T-cells that expressed intracellular IL-10, in the spleen, which, nevertheless, were still significantly higher than that observed in vehicle-treated animals (Fig. 4C and D). The percentages of splenic CD4+ T-cells expressing cytokines such as TNF-α (Fig. 4E), IL-17 (Fig. 4F) and IFN-γ (data not shown) were comparable to those observed in vehicle-or rFVIIIFc-treated animals. Conversely, at 250 IU/kg, both mutants failed to induce tolerance markers over the background observed in control mice (Fig. 4C and D). While rFVIIIFc and rFVIIIFc-IHH showed significantly higher percentages of splenic T-cells producing proinflammatory cytokines (IL-17, TNF-α and IFN-γ), rFVIIIFc N297A did not increase proinflammatory cytokine positive T-cells to levels above that observed in vehicle-treated mice at a dose of 250 IU/kg (Fig. 4E and F and data not shown). Together, these studies suggest a role for both FcγR and FcRn in the tolerogenic pathways observed with rFVIIIFc.

4. Discussion

Replacement therapy with recombinant proteins, though beneficial to most patients, may be hampered by the formation of anti-drug antibodies, counteracting the effectiveness of treatment [26]. FVIII replacement therapy for hemophilia A is an example, where ~30% of patients develop neutralizing antibodies. In addition to regimens aiming to achieve immune tolerance induction, there are initiatives that seek to minimize the immunogenicity potential of FVIII. Herein, we report that rFVIIIFc, which was developed to prolong the circulating half-life of FVIII by genetic fusion of BDD-FVIII and the Fc portion of human IgG1, diminished the antibody response to FVIII in a mouse model of hemophilia A. In addition, our results suggest that the reduced immunogenicity is due to the establishment of a tolerogenic microenvironment in the spleen of HemA mice at therapeutically relevant doses of rFVIIIFc and dependent upon the Fc domain. Consistent with our findings in the preclinical animal model, it is of interest to note that immune tolerance induction with rFVIIIFc has successfully eradicated anti-FVIII inhibitors in 3 children with high antibody titers (peaked at 16-422 BU), including 1 child with the highest titer who previously failed ITI with rFVIII (Lynn M. Malec et al., Abstract from the 57th Annual Meeting of the American Society of Hematology 2015).

The concept of IgG as a tolerogenic carrier and as a means to reduce the antigenicity of the cargo has been demonstrated several decades ago [27,28]. Apart from half-life extension, Fc fusion proteins are also associated with immunomodulatory properties that result in the inhibition of immune responses to linked antigens [29,30]. For example, a fusion protein composed of the IgG1 heavy chain linked with the interphotoreceptor retinoid binding protein (IRBP) antigen, expressed in B-cells, was able to protect mice from experimental autoimmune uveitis directed at the IRBP antigen [31]. Similarly, tolerance to FVIII was also demonstrated by engineering fusion proteins that contained the C2 or A2 domains of FVIII together with IgG [20]. It was recently demonstrated that Fc-mediated transplacental delivery of immunodominant FVIII domains fused with the Fc region of IgG induced tolerance to FVIII [18]. In the present study, we demonstrated that the Fc domain is responsible for suppressing the immune response towards rFVIII, a highly immunogenic protein in mice, at therapeutic doses (see Fig. 5).

Specific polymorphisms within the TNF-α and IL-10 genes have been linked to a higher incidence of inhibitor formation in patients [7,8]. Although higher IL-10 levels have been correlated with increased antibody formation in humans, the presence of splenic IL-10-positive T-cells has also been associated with tolerogenic pathways in HemA mice [10]. Similarly, higher levels of secreted IL-10 and TGF-β and lower levels of pro-inflammatory cytokines such as IL-17 have been demonstrated to extend tolerance to rFVIII in animal models [11]. In our study, rFVIIIFc treatment resulted in a percentage of splenic TNF-α + CD4+ T cells that was similar to untreated animals and significantly lower than that observed in mice that received similar doses of BDD-rFVIII and FL-rFVIII. In contrast, splenocytes from mice receiving therapeutically relevant doses of rFVIIIFc showed upregulation in mRNA levels of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-35. Thus, therapeutically relevant doses of rFVIIIFc prevented the expression of inflammatory cytokines and promoted the expression of cytokines associated with tolerance.

Furthermore, upregulation of tolerance-related markers such as Foxp3, CD25, PD-1 (CD279), and CTLA-4 was identified. Together with the cytokine changes, these data suggest the induction of functional tolerance pathways mediated by regulatory T cells with therapeutically relevant doses of rFVIIIFc (see Fig 3). Treg cells have been shown to induce tolerance to self-antigens as well as to injected protein therapeutics, including FVIII in murine models of hemophilia [32]. Naturally occurring as well as induced splenic regulatory T cells were able to block antibody responses to FVIII in HemA mice [33,34]. CD4+Foxp3+ Treg cells, along with higher percentages of cells positive for surface markers such as CD25 and CTLA-4, were also responsible for immune tolerance to FVIII induced by plasmid-mediated FVIII gene therapy in HemA mice [35]. In another model, rapamycin-induced Treg cells were capable of imparting tolerance to rFVIII in HemA mice. The immune tolerance pathways in this rapamycin-induced model showed phenotypic evidence of Treg cell induction along with up-regulation of TGF-β, CTLA-4 and CD25, but down-regulation of IL-2, IL-4, IL-6, and IL-10 transcripts in splenocytes [13].

Key molecular determinants of tolerogenic dendritic cells [36] observed in our investigations included PD-L1 and IDO-1. IDO-1 is a central tolerogenic molecule activated in dendritic cells that can skew an immune response towards tolerance [36]. In a previous report, transposon based co-delivery of IDO-1 and FVIII genes attenuated inhibitor formation in HemA mice [37]. Our results support the notion that therapeutically relevant doses of rFVIIIFc promote multiple cell types that lead to suppression of immunogenicity and promoting tolerance to FVIII.

The impact of interactions with either FcRn using the nonbinding mutant, rFVIIIFc-IHH, or with FcγR, using the nonbinding mutant rFVIIIFc N297A, were investigated. Despite the lack of binding to these receptors, both mutant FVIII proteins retained reduced immunogenicity at low doses in comparison to FL-rFVIII and BDD-rFVIII. However, both mutants exhibited a markedly attenuated induction of tolerogenic molecules implicating them in the tolerogenic pathways at therapeutic doses, although the levels of these molecules were still significantly higher than those observed in vehicle-treated mice. Conversely, at high doses, both rFVIIIFc and rFVIIIFc-IHH were comparably immunogenic, partly due to an increase in the levels of pro-inflammatory cytokines and lack of induction of Treg cells, whereas preventing the FcγR interaction with the N279A rFVIIIFc tempered the production of pro-inflammatory cytokines although it was not sufficient to induce tolerance. The results suggest that the tolerogenic pathways activated by rFVIIIFc may involve a combination of these two signaling mechanisms, i.e., via FcRn and/or FcγR, presumably FcγRIIb, and potentially others such as the presence of Tregitopes within Fc [19].

The FcγRIIb receptor is an immunosuppressive Fc receptor that has been demonstrated in multiple systems to counteract stimulatory signals [38]. The N297A mutation in the Fc region of IgG abolishes the interaction of Fc with the FcγRIIb receptor. FcγRIIb signaling and induction of CD4+Foxp3+ cells have been reported in the establishment of oral and mucosal tolerance to foreign antigens in mice [39,40], and FcγRIIb-deficient mice exhibit diminished Treg induction against foreign antigens in vivo [40]. These studies have implicated a role for both B-cells and dendritic cells which are known to harbor this immunomodulatory receptor [39,40]. Moreover, co-culture of dendritic cells derived from

FcγRIIb knockout mice, with CD4+ T-cells led to higher proliferation and pro-inflammatory IFN-γ and IL-2 secretion *in vitro* [40]. In agreement with these results, our studies showed attenuated Treg marker levels in low dose N297A treated mice. Of interest, mice treated with high doses of rFVIIIFc N297A did not result in higher levels of splenic T-cells expressing pro-inflammatory cytokines as did rFVIIIFc, likely due to the lack of interaction of rFVIIIFc N297A with stimulatory Fcγ receptors, suggesting the contribution of T-cell cytokines to the immunogenicity observed with high doses of rFVIIIFc.

5. Conclusion

In summary, rFVIIIFc proved to be less immunogenic than FL-rFVIII and BDD-rFVIII at therapeutically relevant doses and promoted the development of phenotypic Tregs and a tolerogenic microenvironment in the spleen of HemA mice. Mechanistically, this tolerogenic effect is partly mediated by the Fc receptors Fcγ and FcRn, which may act in concert with other elements. In contrast, at high doses there was a loss of these tolerogenic properties, owing, at least in part, to the presence of inflammatory cytokines. Moreover, pretreatment with therapeutically relevant, tolerogenic doses of rFVIIIFc resulted in blunting of the immune response to high doses of rFVIIIFc. Our studies therefore not only provide support for the ability of rFVIIIFc to reduce immunogenicity and induce functional tolerance but also may help guide the evaluation of future treatments of rFVIIIFc in hemophilia A patients that aim to prevent immune responses to FVIII.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2015.12.008.

Abbreviations

FVIII	factor VIII
rFVIIIFc	recombinant human factor VIII Fc
BDD	B-domain deleted
FL-rFVIII	full length recombinant factor VIII

Treg	regulatory T-cells
TNF-α	tumor necrosis factor-α
IFN-γ	Interferon-γ

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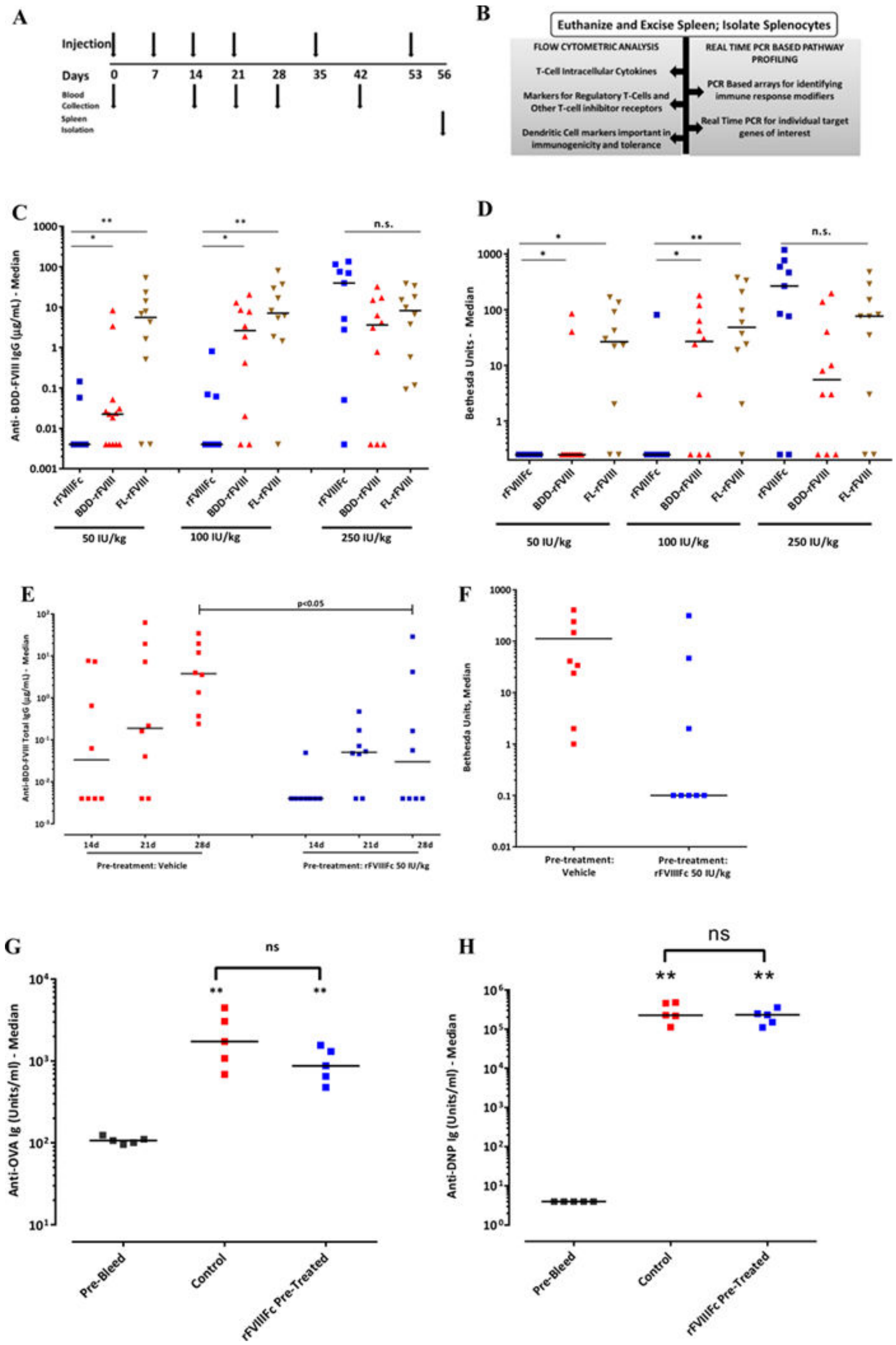

Fig. 1.

rFVIIIFc induces immune tolerance to FVIII. A. Schematic for dosing regimen and analysis. HemA mice (8–10 weeks old) were injected with rFVIIIFc, BDD-rFVIII (Xyntha), FL-rFVIII (Advate) or vehicle, at 50, 100, or 250 IU/kg once weekly for 4 weeks (days 0, 7, 14, and 21) followed by two injections 2 weeks apart (days 35 and 53). Blood was collected by retro-orbital bleeding on days 0, 14, 21, 28, and 42, prior to the dosing, for isolating plasma and determining anti-BDD-FVIII total and neutralizing antibody levels. On day 56, animals were sacrificed and spleens isolated to prepare single splenocyte suspensions. (B)

Splenocytes were subjected to both FACS analysis and PCR-based gene expression profiling as specified. (C) Total anti-BDD-FVIII IgG levels (μg/ml) in individual animals determined on day 42 ($n = 8$–13/group) (D) Neutralizing antibody titers in individual animals on day 42 as determined using the Bethesda assay ($n = 8$–13/group). (E) HemA mice pretreated with 50 IU/kg of rFVIIIFc or vehicle, were rechallenged with 250 IU/kg of rFVIIIFc on day 49, as described in Methods. Results presented are the total anti-FVIII IgG levels (μg/ml) determined on indicated days ($n = 8$/group) (F) Neutralizing antibody titers (BU) on day 28 in rechallenged mice ($n = 8$/group). (G and H) HemA mice pretreated with 50 IU/kg of rFVIIIFc were challenged with DNP-OVA in adjuvant (see Section 2) subcutaneously on days 42 and 49. Results presented are anti-OVA (G) and anti-DNP (H) immunoglobulin levels (units/mL) compared to naïve mice receiving the two injections (control) and pre-bleeds of rFVIIIFc-tolerized mice ($n = 5$/group). The bar represents the median for each treatment group. *$p < 0.05$; **$p < 0.01$; n.s. not significant by Mann–Whitney's T-test.

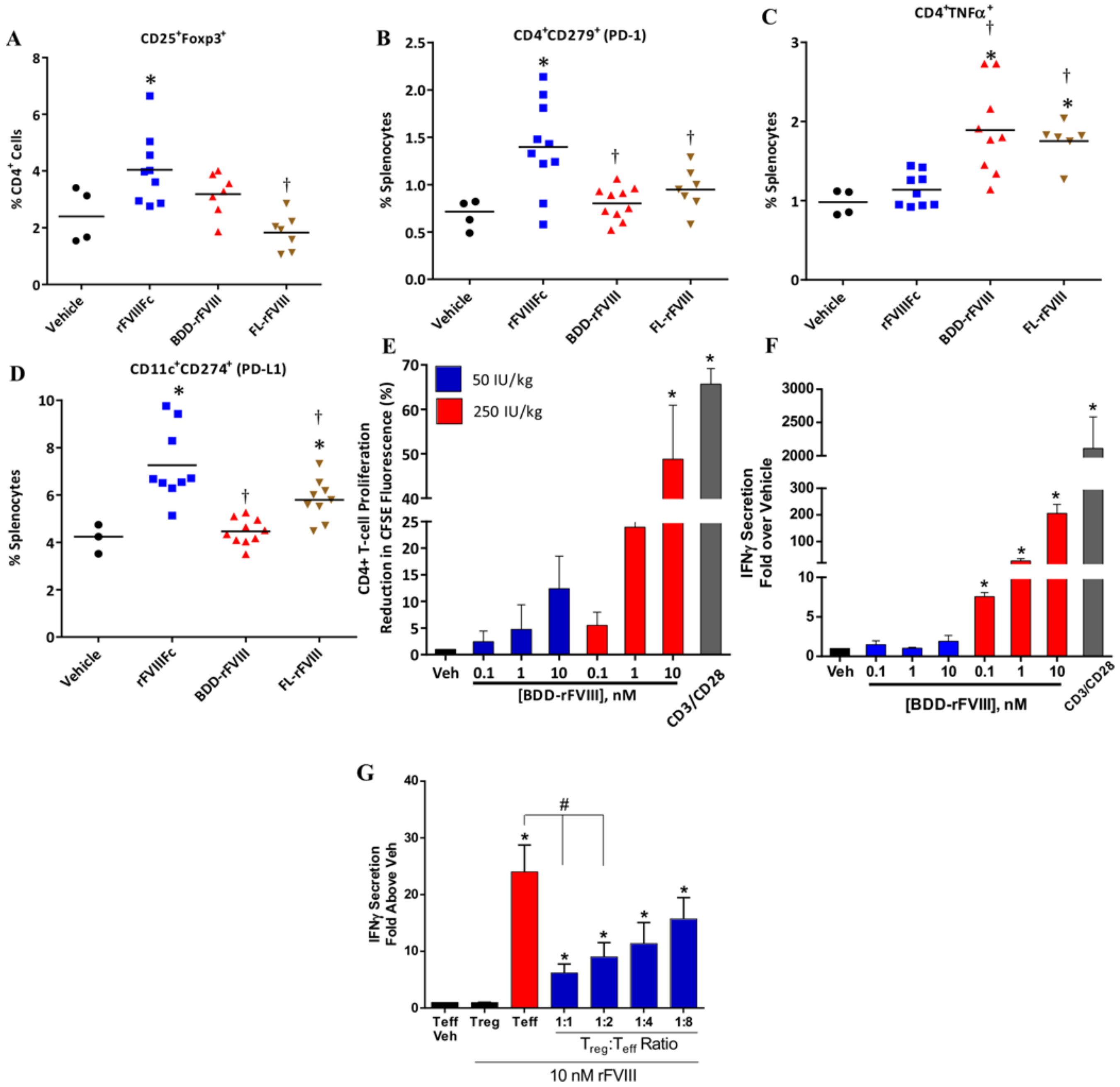

Fig. 2.

rFVIIIFc induces Tregs and associated markers of tolerance. (A) Splenocytes from the 100 IU/kg group were stained for surface CD4 and CD25 followed by intracellular Foxp3 and subjected to FACS analysis. Results represent percent splenocytes positive for CD4, CD25, and Foxp3 ± SEM ($n = 7–9$; $^*p < 0.05$ vs. vehicle; $^†p < 0.05$ vs rFVIIIFc; T-test). (B) CD279 (PD-1) surface staining was determined by co-staining splenocytes from the 100 IU/kg groups with anti-CD279 and anti-CD4 and FACS analysis. Results represent percent of CD4+C279+ splenocytes ± SEM ($n = 7–9$; $^*p < 0.05$ vs. vehicle; $^†p < 0.05$ vs rFVIIIFc; T-test). (C) Splenocytes from mice were co-stained for CD4 and intracellular cytokine TNF-α. Results are percent splenocytes double positive for CD4 and TNF-α ± SEM ($n = 6–10$; $^{*}p < 0.05$ vs. vehicle; $^{\dagger}p < 0.05$ vs rFVIIIFc; *T*-test). (D) Dendritic cell surface expression of CD274 (PD-L1) was determined by staining splenocytes from the 100 IU/kg group for CD274 along with CD11c and MHC Class II ($n = 7–9$; $^{*}p < 0.05$ vs. vehicle; $^{\dagger}p < 0.05$ vs rFVIIIFc; *T*-test). (E) T-cell proliferation was measured using CFSE dye based dilution and FACS. CD4+ T-cells from splenocytes of mice injected with 50 or 250 IU/kg of rFVIIIFc twice, one week apart, were loaded with CFSE and incubated with peritoneal macrophages collected from naïve HemA mice at indicated concentrations of BDD-rFVIII as shown for 96 h at 37 °C. Proliferation was measured as a function of decrease in CFSE MFI. Bars represent decrease in MFI of CFSE relative to vehicle in T-cells ± SEM ($^{*}p < 0.05$, *T*-test, $n = 3–5$). The anti-CD3/CD28 incubations were carried out on CD4+ T-cells derived from the 50 IU/kg group. (F) IFNγ secretion profile from the proliferation studies was measured by ELISA using a MSD (meso scale device) ELISA kit. Bars represent fold above vehicle of IFNγ secretion ± SEM ($^{*}p < 0.05$, *T*-test; $n = 3–5$). (G) CD4+ effector T cells (T_{eff}) and Treg cells were isolated and reconstituted *in vitro* at indicated ratios in the presence of antigen presenting CD90.2^{-} cells (see Section 2). IFNγ secretion was measured by ELISA using a MSD (meso scale device) ELISA kit. Bars represent fold above vehicle of IFNγ secretion ± SEM ($^{*}p < 0.05$ vs vehicle; $^{\#}p < 0.05$ vs T_{eff}, *T*-test; $n = 4$).

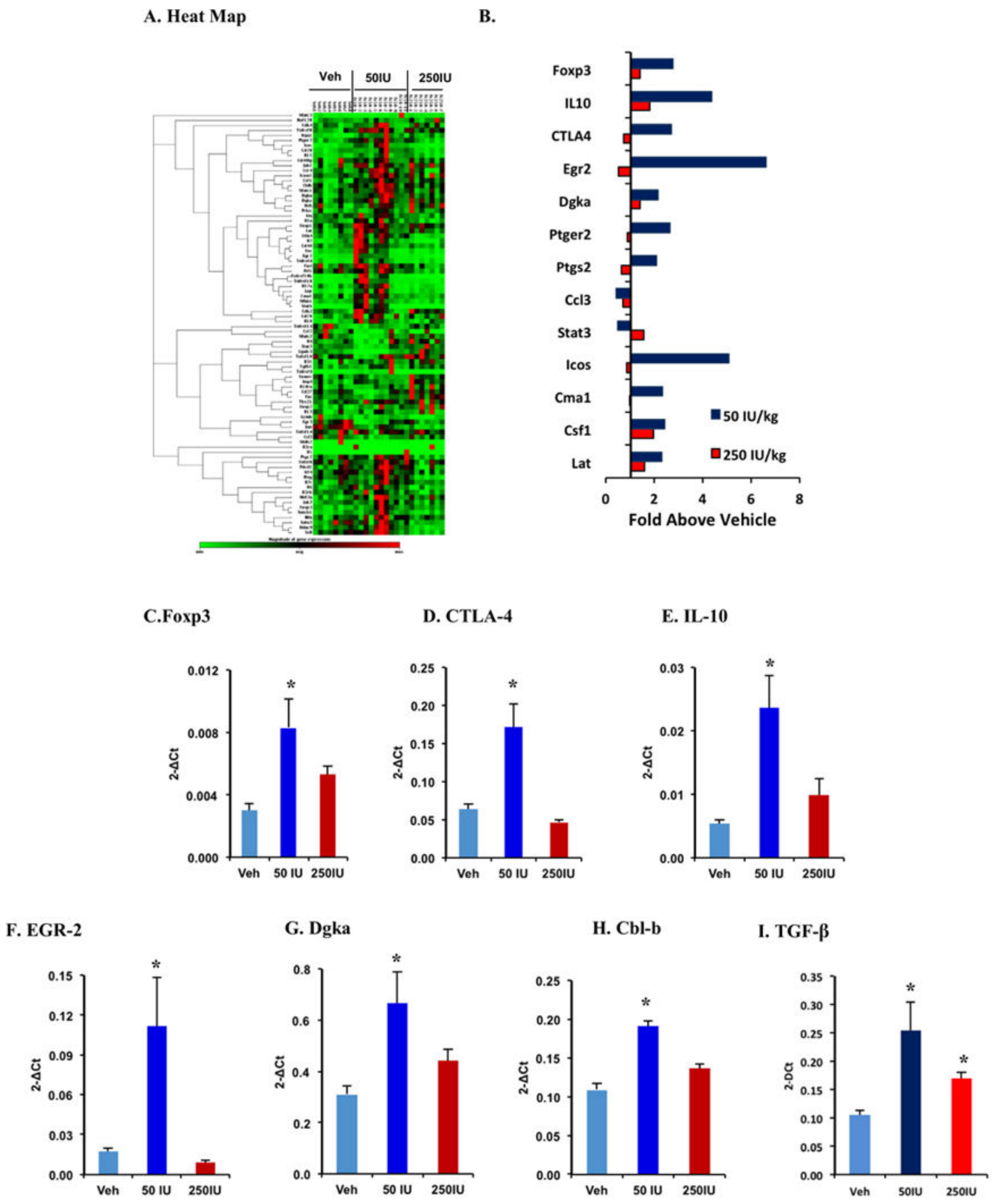

Fig. 3.

Tolerogenic mechanisms activated by rFVIIIFc: (A) heat map depicting the expression profiles of all the genes in the real time PCR array among the three tested groups: vehicle, 50 IU/kg and 250 IU/kg of rFVIIIFc. cDNA from each of the total splenocyte samples was used to monitor the expression of individual genes using a real time PCR array consisting of genes focused on tolerance and anergy associated molecules (*n* = 8–11/group). (B) Expression profile of candidate genes that were identified as being up- or down-regulated by the 50 IU/kg group in comparison with the 250 IU/kg group. Results shown here illustrate the fold change in expression of genes above vehicle group. The cut-off for fold change in regulation was taken as 2, i.e., fold change above 2 was considered up-regulation and below 0.5 as down-regulation. All the candidate genes belonging to the 50 IU/kg group shown here were significantly regulated ($p < 0.05$ vs. vehicle as well as the 250 IU/kg group; $n = 8–11$). Expression levels of some of the candidates (C–H) are confirmed by real time PCR. (I) Real time PCR was carried out to determine levels of TGF-β mRNA transcript. Bars represent $2^{-\Delta Ct}$ values for the three treatments ($p < 0.05$ vs. vehicle as well as the 250 IU/kg group; $n = 8–11$).

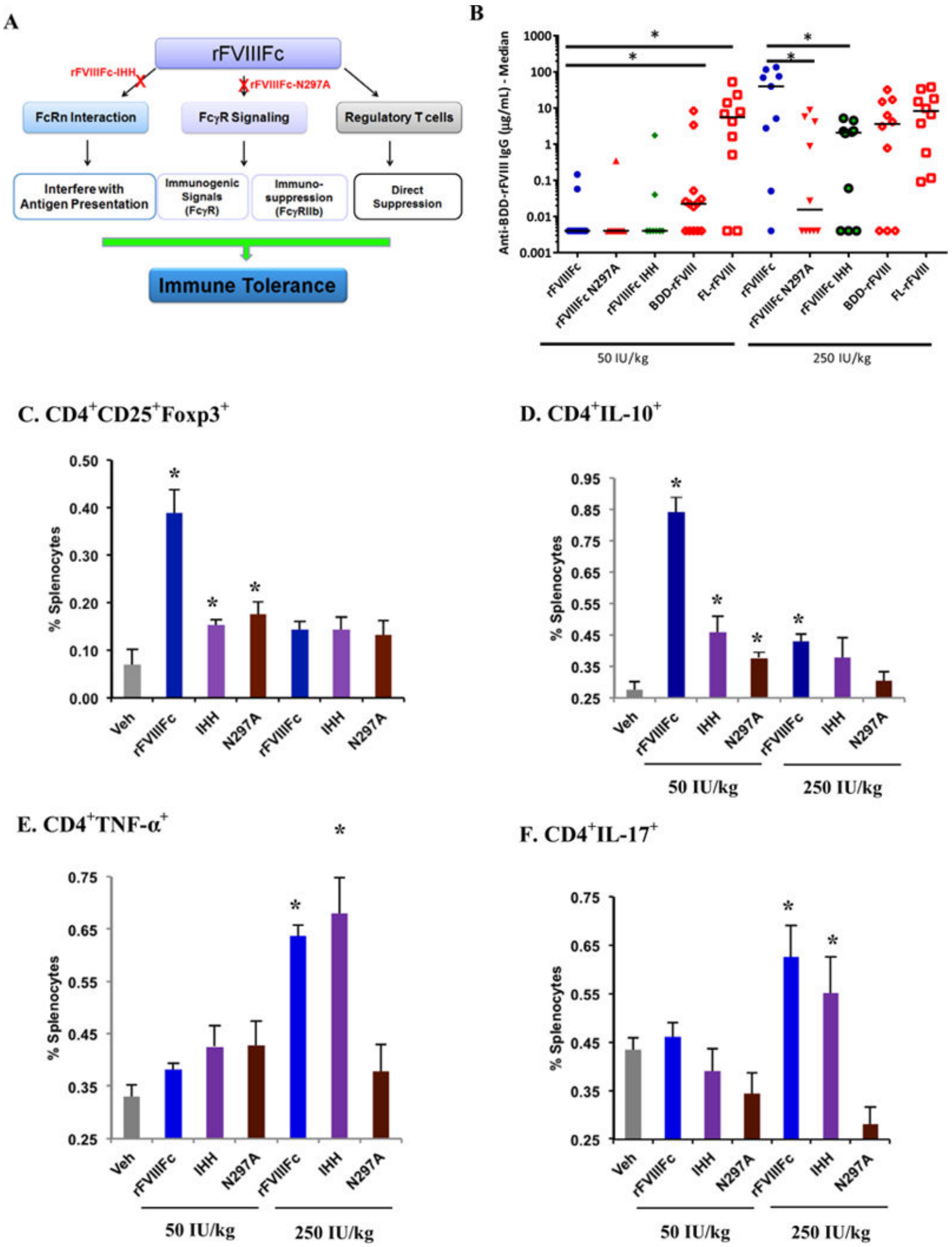

Fig. 4.

rFVIIIFc signals via FcRn and/or Fcγ receptors to induce immune tolerance to rFVIII. (A) Hypothesis for the possible receptor dependent mechanisms for rFVIIIFc to induce tolerance. (B) Total anti-FVIII IgG levels on day 42 in HemA mice injected with 50 or 250 IU/kg of rFVIIIFc, rFVIIIFc-N297A and rFVIIIFc-IHH, in comparison with BDD-rFVIII (Xyntha®) or FL-rFVIII (Advate®). Results illustrated here are anti-BDD-FVIII IgG levels (μg/ml) and the median bar is depicted for each group in the study ($n = 8–13$; *$p < 0.05$; **$p < 0.01$; Mann–Whitney test). (C–F) FACS analysis for markers indicated from splenocytes of mice injected with 50 or 250 IU/kg of rFVIIIFc or mutants. Bars depict % splenocytes for the marker tested + S.E.M. (*$p < 0.05$; *T*-test; $n = 6–11$).

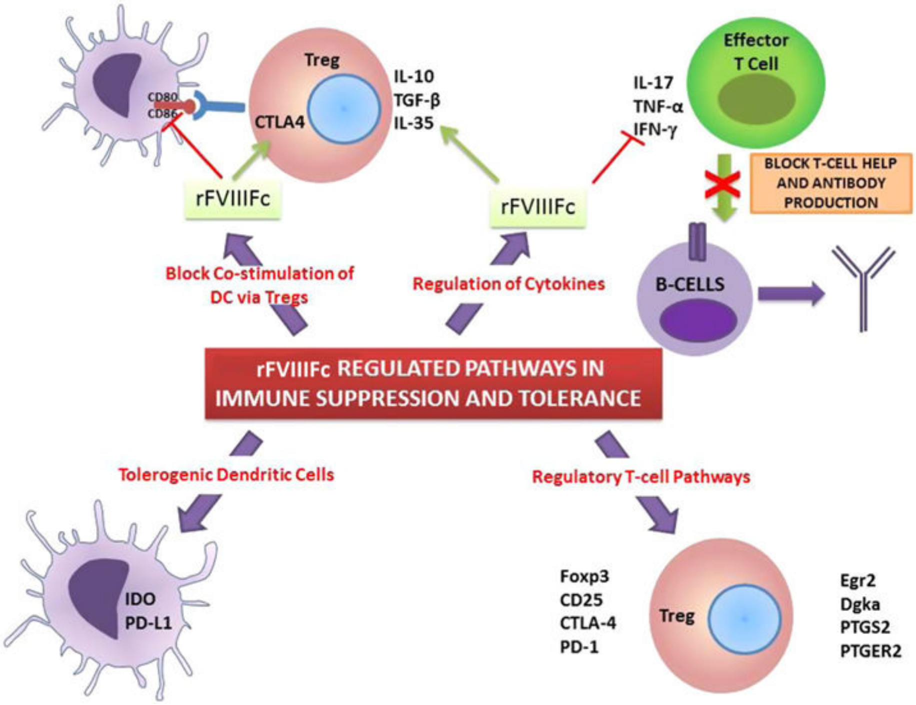

Fig. 5.

Working model for mechanism of action of rFVIIIFc in induction of immune tolerance to rFVIII.