Dexamethasone promotes durable factor VIII-specific tolerance in hemophilia A mice via thymic mechanisms

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ABSTRACT

The development of inhibitory antibodies to factor VIII is the most serious complication of replacement therapy in hemophilia A. Activation of the innate immune system during exposure to this protein contributes to inhibitor development. However, avoidance of factor VIII exposure during innate immune system activation by external stimuli (e.g., vaccines) has not been consistently shown to prevent inhibitors. We hypothesized that dexamethasone, a drug with potent anti-inflammatory effects, could prevent inhibitors by promoting immunologic tolerance to factor VIII in hemophilia A mice. Transient dexamethasone treatment during ainitial factor VIII exposure reduced the incidence of anti-factor VIII immunoglobulin G in both a conventional hemophilia A mouse model (E16KO, 77% *vs.* 100%, *P*=0.048) and a hemophilia A mouse model with a humanized major histocompatibility complex type II transgene (E17KO/hMHC, 6% *vs.* 33%, *P*=0.0048). More importantly, among E17KO/hMHC mice that did not develop anti-factor VIII immunoglobulin G after initial exposure, dexamethasone-treated mice were less likely to develop a response after re-exposure six (7% *vs.* 52%, *P*=0.005) and 16 weeks later (7% *vs.* 50%, *P*=0.097). Similar results were obtained even when factor VIII re-exposure occurred in the context of lipopolysaccharide (30% *vs.* 100%, *P*=0.069). The ability of these mice to develop immunoglobulin G to human von Willebrand factor, a structurally unrelated antigen, remained unaffected by treatment. Transient dexamethasone administration therefore promotes antigen-specific immunologic tolerance to factor VIII. This effect is associated with an increase in the percentage of thymic regulatory T cells (12.06% *vs.* 4.73%, *P*<0.001) and changes in the thymic messenger ribonucleic acid transcription profile.

Introduction

Neutralizing antibodies (inhibitors) against factor VIII (FVIII) develop in approximately 30% of treated severe hemophilia A (HA) patients, remaining the major complication of therapy in this disease.[1] The gold standard for eliminating inhibitors, immune tolerance induction (ITI), is difficult to administer, incompletely effective[2] and expensive.[3] Strategies for preventing inhibitors are therefore needed. The risk of developing inhibitors is not completely predicted by known patient-related genetic risk factors (e.g., *f8* genotype,[4] polymorphisms in *Il10, Ctla4, Tnfa*, major histocompatibility complex class II [MHCII][5]), suggesting that inhibitor risk is modifiable.

Inhibitors are high-affinity immunoglobulin (Ig) G antibodies that are the result of cognate interactions between FVIII-specific B cells and follicular T helper cells (Tfhs). Tfhs are derived from naïve CD4+ T cells following interactions with mature dendritic cells (DCs).[6] In contrast, the interaction of naïve CD4+ T cells

with immature DCs results in differentiation to tolerance-promoting regulatory T cells (Tregs)[7] or T-cell anergy.[8] DC maturation is induced by pro-inflammatory stimuli (e.g., inflammatory cytokines, engagement of pattern recognition receptors), and as such the "decision" regarding immunologic tolerance to FVIII may depend on whether pro-inflammatory stimuli are present during a patient's initial exposure to FVIII.

Inhibitor risk might be reduced by avoiding pro-inflammatory stimuli during initial exposures to FVIII.[9] Patients whose first exposure is in the context of prophylactic rather than on-demand therapy may have a lower inhibitor risk.[10,11] However, it is not always possible to choose the conditions of first exposure to FVIII, since bleeding that requires treatment may occur before the initiation of prophylaxis. Avoiding FVIII exposure in the presence of other clinically-defined pro-inflammatory stimuli (e.g., febrile illness, vaccines, tissue injury) has been suggested to reduce inhibitor risk in an observational study,[9] but these results have not been reproduced. Furthermore, this approach may be difficult to implement,[11] making passive avoidance of innate immune stimulation impractical and ineffective.

Active pharmacologic suppression of inflammatory signals during initial FVIII exposure would be a more controllable strategy. However, the pro-inflammatory signals responsible for FVIII immunogenicity in HA have not been conclusively identified and therefore cannot be specifically targeted. Glucocorticoids, which affect both innate and adaptive immunity, may mediate the suppression of a variety of pro-inflammatory signals and their immunological consequences.[12,13] Therefore, glucocorticoids such as dexamethasone (Dex), are attractive candidates for the suppression of inflammatory danger signals in the context of HA inhibitor development.

To test the ability of Dex to promote immunologic tolerance to FVIII and investigate possible mechanisms of action, we used two murine models of HA. The first model is a severe HA mouse (knockout of exon 17 of the *f8* gene) in which the murine MHCII loci were replaced with a single transgene for a chimeric human/murine MHCII allele (E17KO/hMHC). Approximately 30% of these mice develop antibodies to human FVIII after repeated exposure,[14] suggesting that tolerance is possible, and perhaps inducible, in this model. The second model is a conventional severe HA mouse (knockout of exon 16 of the *f8* gene) in which recombinant human FVIII exposure is immunogenic in 100% of animals (E16KO).[15]

We first hypothesized that E17KO/hMHC mice treated with Dex during an intense initial exposure to FVIII that did not subsequently develop anti-FVIII IgG would, on re-exposure to FVIII, be less likely to develop anti-FVIII IgG than would anti-FVIII IgG-negative mice that were initially treated with FVIII alone. We then sought to determine if our treatment protocol could attenuate the anti-FVIII immune response in E16KO mice and investigate potential cellular mechanisms of action.

Methods

Animals

E17KO/hMHC. HA mice with all murine MHCII alleles knocked out and expressing a single chimeric human/murine transgene of the HLADRB1*1501 allele on a mixed C57Bl6/S129 background. Male mice aged 10-14 weeks were used.[14]

E16KO. HA mice on a homogeneous C57Bl6 background. Mice were sex-matched across treatment groups and eight weeks of age.[16] All animal procedures were in accordance with the Canadian Council on Animal Care guidelines and approved by the Queen's University Animal Care Committee.

Treatment dosing and blood sampling

Dex (Omega) (75µg/dose, ~3mg/kg) was administered intraperitoneally (IP). Recombinant human FVIII (Advate; Baxalta) (6IU/dose, ~240IU/kg unless stated otherwise), lipopolysaccharide (LPS; InvivoGen) (2µg/dose, ~8mg/kg) and ultra-pure plasma-derived human von Willebrand Factor (VWF; Biotest) (2IU/dose, ~80IU/kg) were administered intravenously (IV), *via* tail vein. Hank's balanced salt solution (HBSS) was administered as vehicle control at 100µl IP and 250µl IV.

Intermittent and final blood samples were obtained *via* retro-orbital plexus and cardiac puncture respectively, then mixed in a 1:10 ratio with 3.2% buffered citrate. Plasma was separated by centrifugation, then stored at -80°C.

Short-term treatment protocol

Initial exposure. At week zero, E17KO/hMHC or E16KO mice received FVIII and Dex (FVIII+Dex group) or FVIII alone (FVIII group) for five consecutive days (Figure 1A,B). At week five, blood samples were collected.

Re-exposure. FVIII and FVIII+Dex E17KO/hMHC mice with no evidence of anti-FVIII IgG following initial exposure received FVIII (FVIII/FVIII group and FVIII+Dex/FVIII group), or FVIII and lipopolysaccharide (LPS; FVIII/FVIII+LPS group and FVIII+Dex/FVIII+LPS group) for three consecutive days (week six, Figure 1A). At week nine, blood samples were collected.

Long-term treatment protocol

Initial exposure. E17KO/hMHC mice received FVIII and Dex (FVIII+Dex group) or FVIII alone (FVIII group) for five consecutive days (week zero, Figure 4). At week four, all mice were sampled.

Intermittent low-dose FVIII exposure and re-exposure. FVIII+Dex mice with no evidence of anti-FVIII IgG were divided into two groups. One group received FVIII for three consecutive days at week 16 (FVIII+Dex/FVIII group). The other group received intermittent exposures to low-dose FVIII (2IU/dose at weeks four, eight and 12) followed by FVIII (6IU/dose) for three consecutive days at week 16 (FVIII+Dex/intFVIII+FVIII group). FVIII mice with no evidence of anti-FVIII IgG received FVIII for three consecutive days at week 16 (FVIII/FVIII group). All mice were sampled before (week 14) and after (week 18) FVIII re-exposure.

Human VWF exposure. Mice received human plasma-derived VWF once weekly at weeks 18 to 21. At week 22, blood samples were collected.

Anti-FVIII IgG ELISA

Anti-FVIII IgG titers were measured *via* enzyme-linked immunosorbent assay (ELISA) as previously described.[17] An optical density (OD) cutoff of 0.3 above the OD_{490} of the blank sample was the criterion for positivity, and the titer was determined to be the highest dilution at which a given sample was positive. Samples with an OD_{490} below the cutoff at a 1:40 dilution were considered to have non-detectable anti-FVIII IgG.

Bethesda assay

FVIII inhibitory activity was measured *via* Bethesda assay as previously described.[18] Residual FVIII activity was quantified using an automated coagulometer (STA Compact, Stago). Inhibitory activity was calculated only for samples with a residual FVIII activ-

ity of between 25% and 75%. The reported inhibitory activity was calculated from the plasma dilution that resulted in a residual FVIII activity closest to 50%. Samples with no evidence of FVIII inhibitory activity or with inhibitory activity <0.4BU/ml when undiluted were considered negative.

Anti-human VWF IgG ELISA

Ninety-six-well plates (4HBX, Immulon) were coated with FVIII free plasma-derived human VWF (0.1IU/ml, ~1μg/ml) (Biotest) overnight at 4°C, then blocked for two hours at room temperature. Plasma samples were diluted to 1:40 and incubated for two hours at room temperature in duplicate. IgG detection was carried out as described for the anti-FVIII IgG ELISA. The OD cutoff for

positivity was the OD_{490} of pooled plasma (1:40 dilution) taken from FVIII deficient mice with no previous exogenous VWF exposure. Samples with an OD_{490} below the cutoff at a dilution of 1:40 were considered to have non-detectable anti-human VWF IgG.

Lymphocyte enumeration studies

E16KO mice received HBSS, Dex, FVIII or FVIII+Dex for five consecutive days. Three days or three weeks after the last injection spleen, thymus and blood were collected. Lymphocyte populations were assessed *via* flow cytometry (MACSQuant Analyzer, Miltenyi) by staining for CD19, CD4, CD8, CD25 and FoxP3 (eBiosciences) as appropriate for the particular organ of origin. Data was analyzed with FlowJoX (Tree Star).
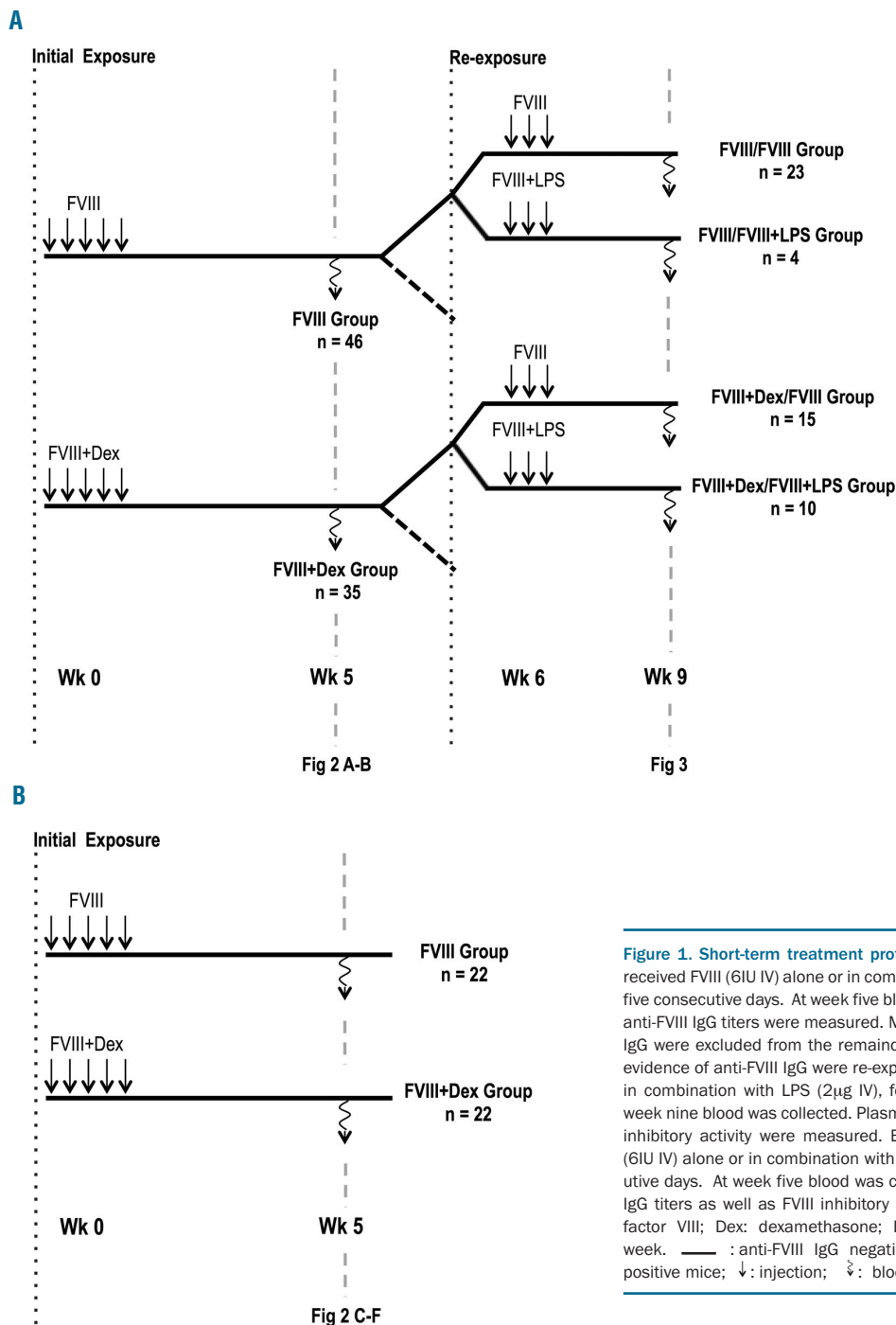

Figure 1. Short-term treatment protocols. A. E17KO/hMHC mice received FVIII (6IU IV) alone or in combination with Dex (75μg IP) for five consecutive days. At week five blood was collected and plasma anti-FVIII IgG titers were measured. Mice with evidence of anti-FVIII IgG were excluded from the remainder of the study. Mice with no evidence of anti-FVIII IgG were re-exposed to FVIII (6IU IV), alone or in combination with LPS (2μg IV), for three consecutive days. At week nine blood was collected. Plasma anti-FVIII IgG titers and FVIII inhibitory activity were measured. B. E16KO mice received FVIII (6IU IV) alone or in combination with Dex (75μg IP) for five consecutive days. At week five blood was collected and plasma anti-FVIII IgG titers as well as FVIII inhibitory activity were measured. FVIII: factor VIII; Dex: dexamethasone; LPS: lipopolysaccharide; Wk: week. ——: anti-FVIII IgG negative mice; - - -: anti-FVIII IgG positive mice; ↓: injection; ↡: blood collection.

Messenger ribonucleic acid (mRNA) expression analysis

E16KO mice received HBSS, Dex, FVIII or FVIII+Dex for five consecutive days. Three days after the last injection, spleen and thymus were collected and stabilized in RNA Later (Invitrogen). mRNA was then isolated using a commercial kit (RNeasy Plus Mini Kit, Qiagen) and quantified using the NanoString Mouse Immunology Panel (NanoString). Data was analyzed with nSolver Software (NanoString). Genes that were deemed up/down-regulated due to FVIII+Dex treatment had a transcript count ratio ≥2:1 in the same direction when comparing both FVIII+Dex against FVIII and Dex against HBSS.

Statistics

Anti-FVIII IgG and FVIII inhibitors incidence were compared using Fisher's exact test. Anti-FVIII IgG and Bethesda titers were compared using a Mann-Whitney U test. E17KO/hMHC samples, but not E16KO samples, with titers below the detection limit were excluded from statistical analyses. Percentages of lymphocyte populations were compared using unpaired two-tailed *t*-tests. Statistical analyses were performed using GraphPad Prism 5.0a (GraphPad Software).
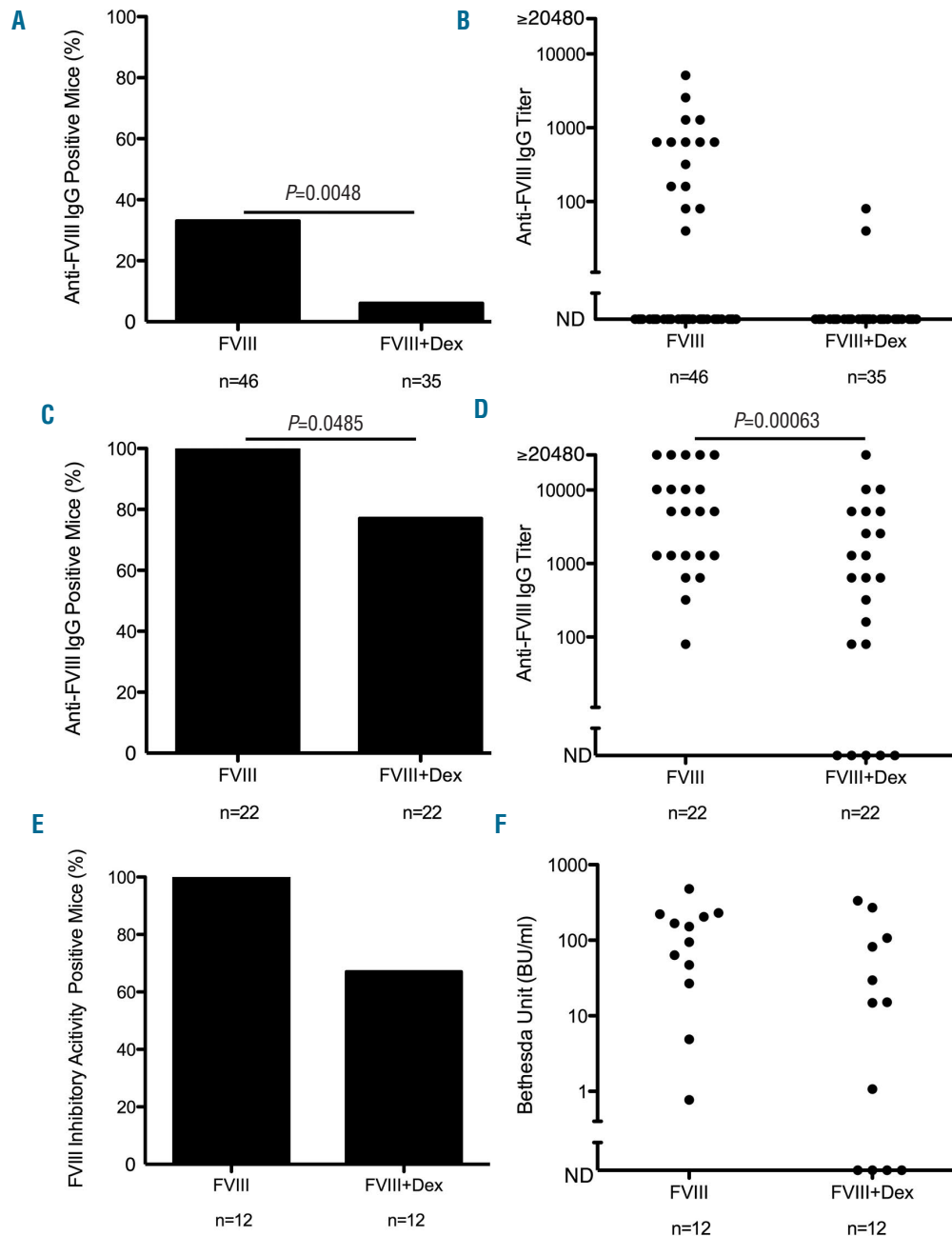

Figure 2. Administration of Dex during initial FVIII exposure reduces the initial anti-FVIII immune response in both E17KO/hMHC and E16KO mice. A. Anti-FVIII IgG incidence and B. anti-FVIII IgG titers in E17KO/hMHC mice five weeks after initial treatment with FVIII or FVIII+Dex. A statistical comparison of the positive anti-FVIII IgG titers across treatment groups could not be performed because fewer than three mice had evidence of antibodies in the FVIII+Dex group. G. Anti-FVIII IgG incidence, D. anti-FVIII IgG titers, E. FVIII inhibitor incidence and F. FVIII inhibitory activity in E16KO mice five weeks after initial treatment with FVIII or FVIII+Dex. ND: not detectable; FVIII: factor VIII; Dex: dexamethasone; IgG: immunoglobulin G.

Results

Administration of Dex during initial FVIII exposure reduces the initial anti-FVIII immune response in both E17KO/hMHC and E16KO mice

Our first aim was to determine the ability of Dex to prevent the anti-FVIII immune response when administered during initial antigen exposure. FVIII was administered alone or in combination with Dex for five consecutive days (week zero, Figure 1A,B). At week five, 6% of E17KO/hMHC FVIII+Dex mice compared to 33% of

E17KO/hMHC FVIII mice had evidence of plasma anti-FVIII IgG (P=0.0050, Figure 2A). Furthermore, FVIII+Dex mice that developed anti-FVIII IgG had lower antibody titers than FVIII mice (Figure 2B). A similar effect was observed in E16KO mice, with 77% of E16KO FVIII+Dex mice compared to 100% of E16KO FVIII mice showing evidence of plasma anti-FVIII IgG (P=0.0485, Figure 2C), and FVIII+Dex mice having significantly lower anti-FVIII IgG titers than FVIII mice (P=0.0063, Figure 2D). Although not statistically significant, a similar trend was observed when looking at inhibitor incidence and activity in E16KO mice (Figure 2E,F).
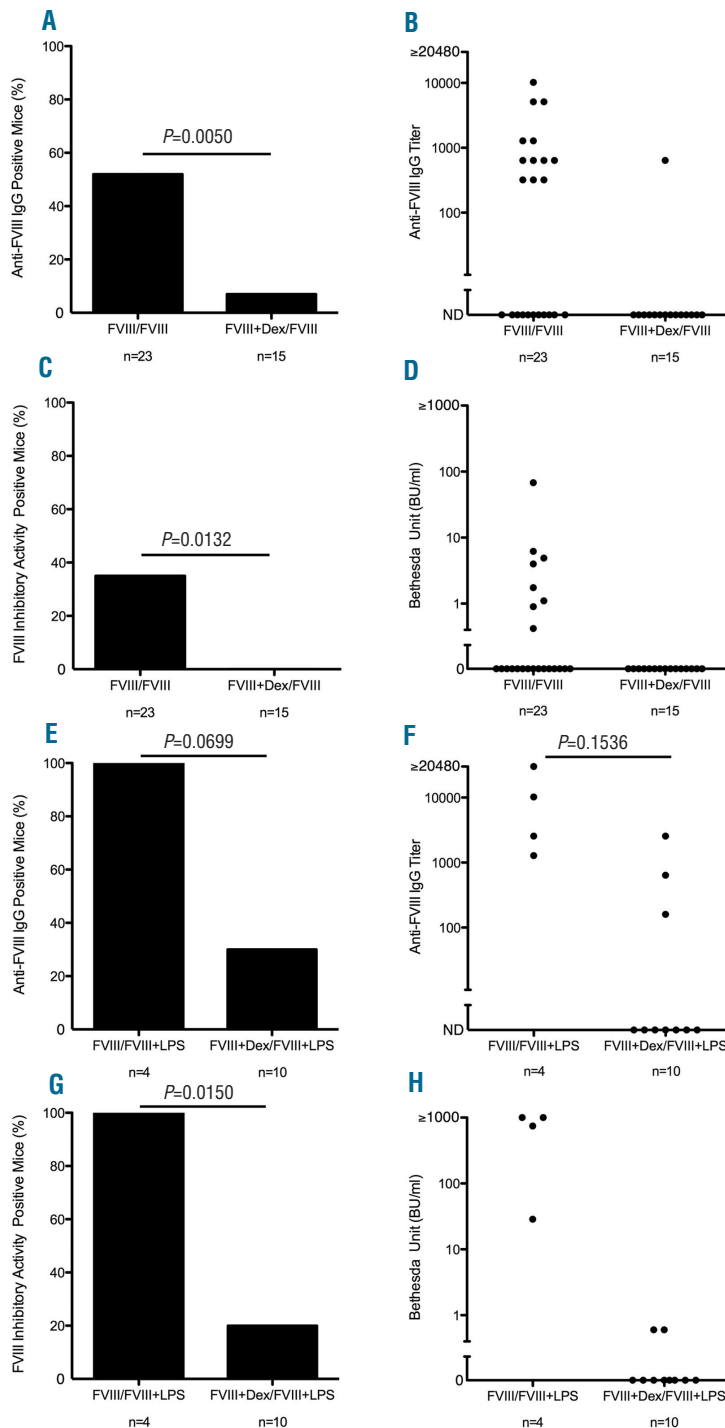

Figure 3. Administration of Dex during initial FVIII exposure induces tolerance to FVIII in E17KO/hMHC mice, even when co-administered with LPS. A. Anti-FVIII IgG incidence, B. anti-FVIII IgG titers, C. FVIII inhibitor incidence and D. FVIII inhibitory activity following re-exposure to FVIII in E17KO/hMHC mice initially exposed to FVIII or FVIII+Dex and with no evidence of anti-FVIII IgG at week five. E. Anti-FVIII IgG incidence, F. Anti-FVIII IgG titers, G. FVIII inhibitor incidence and H. FVIII inhibitory activity following re-exposure to FVIII+LPS in E17KO/hMHC mice initially exposed to FVIII or FVIII+Dex and with no evidence of anti-FVIII IgG at week five. Some statistical comparisons could not be carried out because fewer than three mice had evidence of antibodies and/or inhibitors. FVIII: factor VIII; Dex: dexamethasone; IgG: immunoglobulin G; LPS: lipopolysaccharide; ND: not detectable.

Administration of Dex during initial FVIII exposure induces tolerance to FVIII in E17KO/hMHC mice, even when co-administered with LPS

Next, we wanted to determine whether E17KO/hMHC mice that did not develop anti-FVIII IgG after initial exposure would be immunologically tolerant upon re-exposure to FVIII. Anti-FVIII IgG negative mice from both the FVIII and FVIII+Dex groups were re-exposed to FVIII alone or in combination with LPS (week 6, Figure 1A).

At week nine, 7% of FVIII+Dex/FVIII mice compared to 52% of FVIII/FVIII mice had evidence of plasma anti-FVIII IgG (*P*=0.0050, Figure 3A). The single FVIII+Dex/FVIII mouse with evidence of anti-FVIII IgG had a lower titer than most of the FVIII/FVIII mice (Figure 3B). Furthermore, 0% of FVIII+Dex/FVIII mice compared to 35% of FVIII/FVIII mice showed evidence of FVIII inhibitors by Bethesda assay (*P*=0.0132, Figure 3C,D).

At week nine, 100% of FVIII/FVIII+LPS mice compared to 30% of FVIII+Dex/FVIII+LPS mice had evidence of anti-FVIII IgG (*P*=0.0699, Figure 3E). FVIII+Dex/FVIII+LPS mice also had a trend towards lower anti-FVIII IgG titers than FVIII/FVIII+LPS mice (*P*=0.1536, Figure 3F). Furthermore, 20% of FVIII+Dex/FVIII+LPS compared to 100% of FVIII/FVIII+LPS mice (*P*=0.0150, Figure 3G) had evidence of FVIII inhibitors and FVIII+Dex/FVIII+LPS mice had lower FVIII inhibitor levels than FVIII/FVIII+LPS (Figure 3H). These data suggest that Dex can promote persistent tolerance to FVIII in the E17KO/hMHC murine

model of HA, and that this tolerance is robust enough to withstand re-exposure to FVIII when co-delivered with LPS, a potent adjuvant.

Administration of Dex during initial FVIII exposure induces durable, antigen-specific tolerance to FVIII in E17KO/hMHC mice

We next sought to investigate the durability of Dex-induced tolerance to FVIII, using the long-term treatment protocol described (Figure 4). At week four, 0% of FVIII+Dex E17KO/hMHC mice compared to 64% of FVIII E17KO/hMHC mice had evidence of anti-FVIII IgG (*P*=0.0001, Figure 5A,B). Mice with evidence of anti-FVIII IgG at week four were excluded from the remainder of the experiment. At week 14, all remaining mice had maintained their anti-FVIII IgG negative status. At week 18, two weeks after re-exposure to FVIII, 7% of FVIII+Dex/FVIII mice and 27% of FVIII+Dex/intFVIII+FVIII mice had evidence of anti-FVIII IgG compared to 50% of FVIII/FVIII mice (*P*=0.0970 FVIII/FVIII *vs.* FVIII+Dex/FVIII; *P*=0.5573 FVIII/FVIII *vs.* FVIII+Dex/intFVIII+FVIII; *P*=0.3295 FVIII+Dex/FVIII *vs.* FVIII+Dex/intFVIII+FVIII, Figure 5C). No apparent differences were seen between the titers of the few mice from each group positive for anti-FVIII IgG (Figure 5D). These data indicate that administration of Dex during initial FVIII exposure confers tolerance that persists for at least 18 weeks, and that ongoing intermittent FVIII exposure is
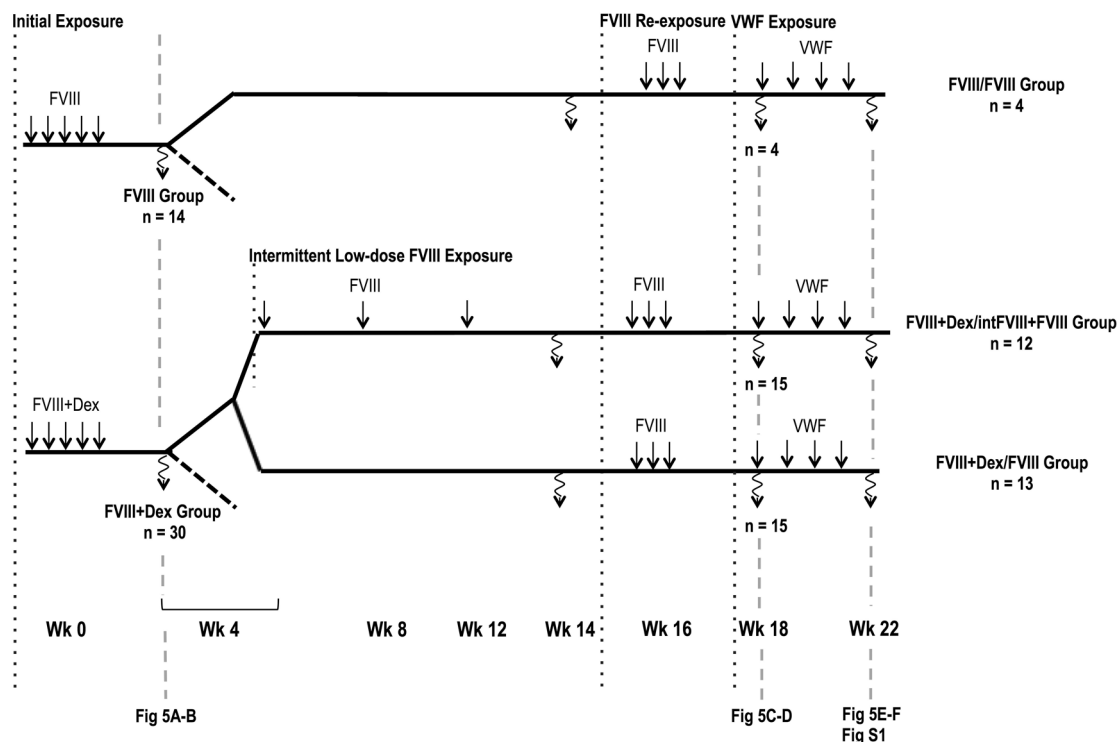

Figure 4. Long-term treatment protocol. E17KO/hMHC mice received FVIII (6IU IV) alone or in combination with Dex (75µg IP) for five consecutive days. At week four, blood was collected and plasma anti-FVIII IgG titers were measured. Mice with evidence of anti-FVIII IgG were excluded from the remainder of the study. FVIII+Dex mice with no evidence of anti-FVIII IgG received FVIII (6IU IV) for three consecutive days at week 16, or single intermittent exposures to low-dose FVIII (2IU IV) at weeks four, eight and 12, followed by FVIII (6IU IV) for three consecutive days at week 16. FVIII treated mice with no evidence of anti-FVIII IgG at week four received FVIII for three consecutive days at week 16. All mice were sampled two weeks before and two weeks after FVIII re-exposure. Plasma anti-FVIII IgG titers were determined at these time points. At week 18 mice started receiving weekly human VWF injections for four consecutive weeks. At week 22, blood was collected and plasma FVIII inhibitory activity and the presence of plasma anti-human VWF IgG were determined. VWF: von Willebrand Factor; FVIII: factor VIII; Dex: dexamethasone; Wk: week. ⊢ anti-FVIII IgG negative mice; ┅ anti-FVIII IgG positive mice; ↓: injection; ⚡: blood collection.

not required to maintain this tolerance.

To determine whether the observed effect is antigen specific, we injected all mice with a structurally unrelated antigen (human VWF) (week 18-21). At week 22, of the anti-FVIII IgG negative mice, 100% of FVIII+Dex/FVIII, 89% of FVIII+Dex/intFVIII+FVIII and 100% of FVIII/FVIII mice had evidence of anti-human VWF IgG (*Online Supplementary Figure S1*). We conclude that Dex treatment during initial FVIII exposure does not result in general immunosuppression but rather promotes antigen-specific tolerance to FVIII, and does not impair immune responses to other antigens.

At week 22, we also measured FVIII inhibitory activity. 8% of FVIII+Dex/FVIII, 8% of FVIII+Dex/intFVIII+FVIII and 50% of FVIII/FVIII mice had evidence of FVIII inhibitors ($P=0.1206$ FVIII/FVIII *vs.* FVIII+Dex/FVIII; $P=0.1357$ FVIII/FVIII *vs.* FVIII+Dex/intFVIII+FVIII; $P=1$ FVIII+Dex/FVIII *vs.* FVIII+Dex/intFVIII+FVIII, Figure 5E). No apparent differences were seen between the titers of the few mice from each group positive for inhibitors (Figure 5F).

Administration of Dex during initial FVIII exposure causes early changes in lymphocyte populations of E16KO mice

To elucidate possible cellular mechanisms of our treatment protocol, we determined the percentage of key lymphocyte populations in the thymus, spleen and blood *via* flow cytometry. Three days after treatment, FVIII+Dex mice had a decreased percentage of both splenic (47.22% *vs.* 53.62%, $P=0.0395$) and blood (18.27% *vs.* 29.11%, $P=0.0050$) B cells (CD19+ lymphocytes) compared to FVIII

mice (Figure 6A). At this time point, no significant changes in the percentages of T cells (CD4$^+$CD8$^-$ lymphocytes) were observed across the three tissues (Figure 6B). However, FVIII+Dex mice showed a significant increase in the percentage of thymic Tregs (CD25 and FoxP3 expressing CD4$^+$CD8$^-$ lymphocytes, 12.06% *vs.* 4.73%, $P<0.0010$, Figure 6C). Similar trends were observed when comparing Dex and HBSS mice (Figure 6A-C). This suggests that Dex promotes tolerance to FVIII partly by decreasing the percentage of splenic and circulating B cells as well as skewing the distribution of lymphocytes in the thymus towards a regulatory phenotype early after treatment.

Three weeks after treatment, FVIII+Dex mice had no significant differences in thymic and splenic lymphocyte populations when compared to FVIII mice (Figure 6D-F). We did however observe an increase in the percentage of blood B cells (36.33% *vs.* 21.33%, $P=0.028$) in FVIII+Dex mice compared to FVIII mice (Figure 6D). No significant differences in thymic, splenic or blood lymphocyte populations were detected when comparing Dex and HBSS mice (Figure 6D-F). This suggests that the effects of Dex likely occur early on and have no major lasting impact on lymphocyte populations despite the long-term FVIII tolerance.

Administration of Dex during initial FVIII exposures alters the thymic but not splenic transcript profile of E16KO mice

We also assessed the effects of our treatment protocol on thymic and splenic mRNA transcription profiles of E16KO mice. In the thymus, a total of 54 genes had
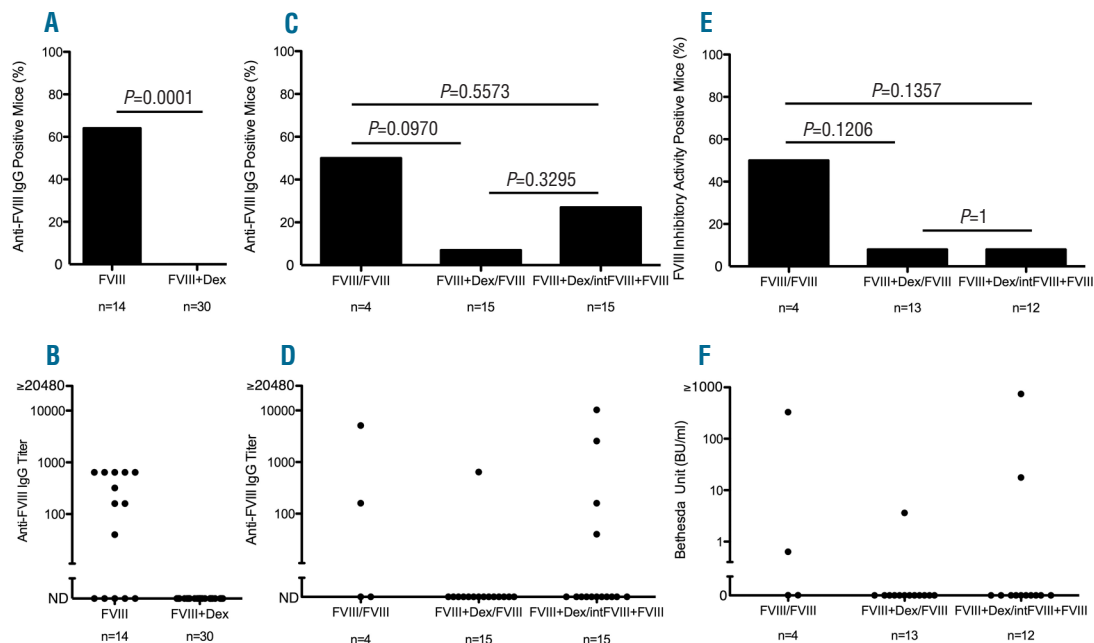

Figure 5. Administration of Dex during initial FVIII exposure induces durable tolerance to FVIII in E17KO/hMHC mice. A. Anti-FVIII IgG incidence and B. anti-FVIII IgG titers following initial exposure with FVIII or FVIII+Dex. C. Anti-FVIII IgG incidence, D. anti-FVIII IgG titers, E. FVIII inhibitor incidence and F. FVIII inhibitory activity following FVIII re-exposure in E17KO/hMHC mice initially treated with FVIII or FVIII+Dex and with no evidence of anti-FVIII IgG at week four. Some statistical comparisons could not be carried out because fewer than three mice had evidence of antibodies and/or inhibitors. ND: not detectable; FVIII: factor VIII; Dex: dexamethasone; IgG: immunoglobulin G.

altered expression due to FVIII+Dex treatment (Figure 7A, *Online Supplementary Table S1* and *Table S2*). There were no differences between the splenic mRNA transcription profiles of FVIII+Dex and FVIII mice (Figure 7B). There were also no differences between the thymic and splenic mRNA transcription profiles of FVIII and HBSS mice (*Online Supplementary Figure S2*).

Discussion

We sought to determine whether Dex, when administered during initial FVIII exposure, could promote immunologic tolerance to FVIII in HA mice. Our experiments indicate that both E17KO/hMHC and E16KO FVIII+Dex mice were less likely to develop anti-FVIII IgG than FVIII mice after initial exposure to FVIII. Although E17KO/hMHC mice can have inherent tolerance to FVIII, the ability of Dex to also reduce inhibitor development in E16KO mice is encouraging. While any immediate effect might have been due to transient immunosuppression, the reduced incidence of anti-FVIII IgG in E17KO/hMHC FVIII+Dex/FVIII mice after re-exposure to FVIII at six, and especially 16, weeks suggests that long-lasting tolerance to FVIII can be promoted. Furthermore, this tolerance is specific to FVIII since these mice mount a robust response to a structurally unrelated antigen (human VWF) despite remote exposure to Dex.

Especially noteworthy is the reduced anti-FVIII immune response after Dex exposure in mice whose re-exposure to FVIII was accompanied by LPS. LPS administration with FVIII has been reported to yield anti-FVIII IgG in 100% of E17KO/hMHC mice,[14] an effect also observed in our experiments. Compared to FVIII/FVIII+LPS mice, FVIII+Dex/FVIII+LPS mice demonstrated a markedly reduced anti-FVIII immune response, although this effect did not reach statistical significance due to small numbers.

We investigated potential cellular mechanisms of Dex-mediated tolerance induction by examining its effect on lymphocyte populations of E16KO mice. Three days after treatment we observed a decrease in the percentage of splenic and circulating B cells. A subset of splenic B cells has been shown to play a role in the initiation of the anti-FVIII immune response.[19] B cells also maintain this response as their inhibition has been identified as a potential mechanism of ITI in mice.[20] Furthermore, in inhibitor patients who fail conventional ITI, the addition of rituximab to target B cells has been shown to increase ITI efficacy.[21] Three days post-treatment we also observed an increase in the percentage of thymic Tregs. This T-cell subset has been repeatedly implicated in tolerance to FVIII in HA mouse models.[18,22,23] Tregs simultaneously interact with antigen-presenting cells and effector T cells, resulting in effector T-cell suppression. The changes in lymphocyte populations following Dex treatment were no longer present three weeks post-treatment.

Our results are in line with previous studies showing that glucocorticoids can induce apoptosis of B and T cells[13] and that Tregs, especially those in the thymus, are preferentially spared from Dex-induced cell death.[24] There is some evidence suggesting that repeated antigen exposure is required for the maintenance of Treg populations.[25] However, in our experiment, FVIII+Dex/intFVIII mice did not maintain tolerance to FVIII better than FVIII+Dex/FVIII mice.

FVIII+Dex mice also had altered thymic gene expression, giving further insight into the mechanism of our treatment protocol. We observed down-regulation of genes involved in T-cell receptor formation and rearrangement (*Cd4, Rag1, Rag2*), genes coding for cytokines that drive effector T-cell proliferation and maturation (*Il9, Il12b, Il13,*[26] *Il16,*[27] *Il27*[28]) and genes responsible for T-cell activation (*Cd40lg,*[29] *Lck*[30]). In contrast, we saw up-regulation of genes encoding for scavenger receptors involved in clearance of apoptotic cells (*Cd36,*[31] *MARCO*[32]) and genes responsible for thymic involution (*Pparg*[33]). We also observed an up-regulation of genes encoding extracellular matrix components (*Fn1*[34]) and adhesion molecules (*Ita2b, Cdh5*) that may play a role in regulating thymocyte development and migration.[35] The splenic gene expression profile in FVIII and HBSS control mice were almost identical, and thus it appears that intense FVIII exposure alone does
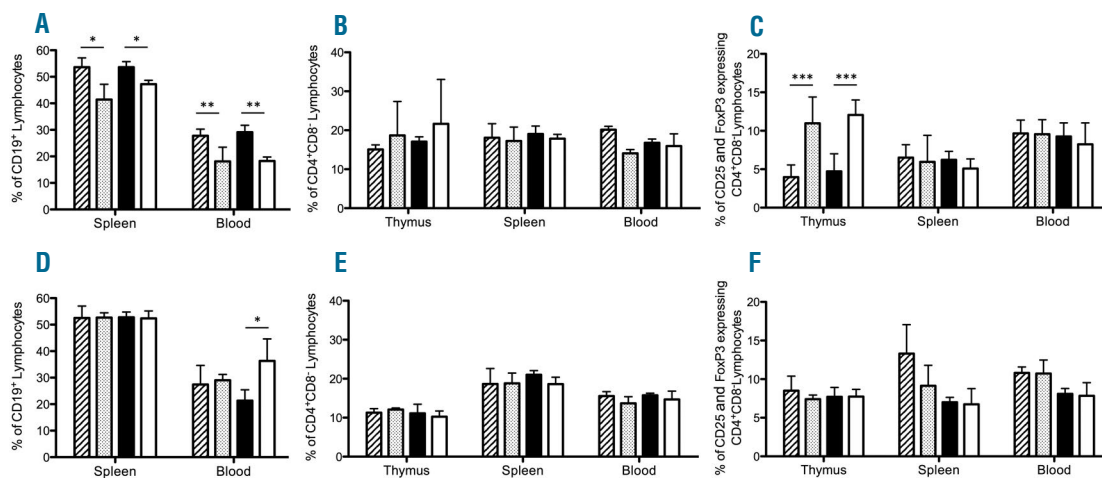

Figure 6. Administration of Dex during initial FVIII exposure causes early changes in lymphocytes populations of E16KO mice. The percentage of A. B cells, B. T cells and C. Tregs in the thymus, spleen and blood three days after treatment with HBSS, Dex, FVIII or FVIII+Dex. The percentage of D. B cells, E. T cells and F. Tregs in the thymus, spleen and blood three weeks after treatment with HBSS, Dex, FVIII or FVIII+Dex. n=3-7 for each condition. *P<0.05, **P<0.01, ***P<0.001. ▨ : HBSS; ▢ : Dex; ▉ : FVIII; ▢ : FVIII+Dex.

not result in altered expression of immune-relevant genes.

A number of prior studies have examined the ability of immunomodulatory agents to prevent FVIII inhibitors in murine models of HA: anti-CD3 monoclonal antibody,[18] anti-CD4 monoclonal antibody with adjuvant,[36] rapamycin,[22] CD40/CD40-ligand interaction blockade,[37] and IL2/ anti-IL2 monoclonal antibody complexes.[38] However, some of these strategies only induced transient tolerance to FVIII[37] or tolerance that was not tested for long-term durability.[18] Furthermore, the effect on immune responses to other antigens was not assessed in some cases.[18,37] Importantly, none of these other immunomodulatory agents is commonly used in clinical practice.

Dex and other glucocorticoids are widely available, routinely used by hematologists and are known to have good oral bioavailability.[39] Glucocorticoids have been used in clinical practice to reduce humoral immune responses to protein therapeutics (e.g., infliximab),[40] and have even been successful in hemophilia patients with inhibitors.[41] Moreover, because the greatest risk of FVIII inhibitors occurs early (~25 first exposure days), clinical application of this approach might only require Dex coverage of a few early FVIII exposures, until the inhibitor risk is reduced. For these reasons, translation of this approach to the bedside seems feasible. A clinical trial examining the use of Dex during early FVIII exposures expected to span several days would be the most immediate application.

This study does have some limitations. As previously mentioned, E17KO/hMHC mice have a mixed genetic background. While this variability between animals may be responsible for some of the observed variability in anti-FVIII immune responses, it may also indicate some biological robustness of the tolerance effect. Due to the genetic variability of E17KO/hMHC, experiments identifying the mechanistic basis of the effect observed were carried out using the inbred E16KO mouse model. The ability of our

treatment protocol to diminish the anti-FVIII immune response in this model with high propensity for inhibitor development further confirms the robustness of the effect.

Although the E17KO/hMHC mouse model of HA recapitulates the epidemiology of anti-FVIII immune responses among humans with severe HA, this does not imply that the immunological mechanisms of these responses are identical. There are significant differences between these species, such as the absence of the IgG4 isotype in mice, which is the dominant IgG subclass associated with inhibitors in HA patients.[42] In addition, this mouse model has only one MHC allele and will therefore have non-physiologic antigen presentation. The age of the model might also be a limitation, as our mice would be considered "young adults". In contrast, HA patients who are at the greatest risk for inhibitor development are toddlers. The effects of these age differences on the ability of Dex to prevent inhibitors in humans cannot be predicted.

Our dose of Dex (~3mg/kg/day, demonstrated to have anti-inflammatory effects in rodents)[43] is higher than that typically used in humans. Small animals require higher per-weight doses of medications than humans to achieve equivalent dosing relative to body surface area.[44] Dex is given to children in doses of 0.3 – 0.6mg/kg/day for the treatment of croup[45] and asthma.[46] In these applications, Dex exerts the anti-inflammatory and anti-lymphocytic effects that may be important for promoting immunologic tolerance to FVIII. Therefore, similar dosing would be reasonable to use in a clinical study addressing the mitigation of FVIII inhibitor development. Although Dex can be immunosuppressive, the risk of invasive infections is low after intermittent exposure in young children.[47]

We also used FVIII doses (~240units/kg/dose) higher than those used in most clinical applications. However, this is the FVIII dose required to provoke FVIII immune responses in E17KO/hMHC mice. Although lower doses
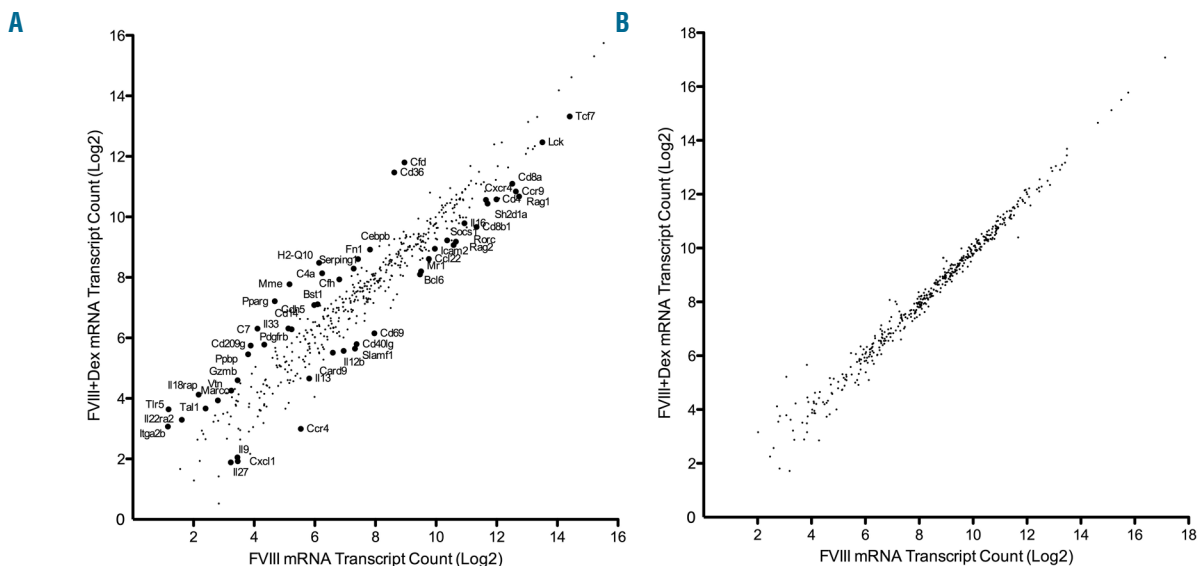

Figure 7. Administration of Dex during initial FVIII exposure alters the thymic but not splenic mRNA transcript profile of E16KO mice. A. Thymic and B. splenic mRNA transcript counts three days after treatment with FVIII+Dex *versus* mRNA transcript counts three days after treatment with FVIII alone. Each point corresponds with the average mRNA transcript count of three different tissue samples. Labeled points indicate genes that exhibited a ≥2-fold change as a result of treatment with FVIII+Dex. These genes had a gene count ratio ≥2:1 in the same direction when comparing both FVIII+Dex against FVIII and Dex against HBSS. n=3 for each condition. FVIII: factor VIII; Dex: dexamethasone; mRNA: messenger ribonucleic acid.

could likely have been used in the E16KO mouse model, we wanted a consistent treatment protocol across the two strains. There is no conceptual reason that lower per-weight FVIII doses would result in evasion of the toler-ance-promoting mechanisms.

The use of high FVIII doses over consecutive treatment days simulated "peak treatment moments" known to be associated with an increased risk of inhibitors.[10] However, this intense initial exposure to FVIII was not given because of a hemorrhagic event, which from a clinical point of view is unrealistic. Patients often receive more intense ini-tial exposures to FVIII to treat life-threatening bleeding, when inflammation may be present and could enhance the immune response to FVIII. We cannot infer that Dex would exert the same effect in these scenarios. However, animal models may be of limited utility in studying these more clinically relevant circumstances. For example, rodent studies have not consistently identified an associa-tion between hemarthrosis[48,49] and anti-FVIII immune responses. Ultimately, clinical studies will be needed to answer the most important questions about the use of Dex in patients with severe HA.

Conclusions

Our experiments show that Dex, when administered during an intense initial exposure to FVIII, diminishes the anti-FVIII immune response in E17KO/hMHC and E16KO HA mice. In addition, this treatment protocol promotes durable and antigen-specific immunologic tolerance to FVIII in E17KO/hMHC mice. This effect appears to be mediated by alterations in lymphocyte populations and thymic gene expression. Clinical studies are needed to determine if this approach can be translated into clinical practice to prevent the devastating occurrence of FVIII inhibitors for which we currently offer no mitigation strat-egy, even in high-risk patients. Ready access to Dex and other glucocorticoids, ease of administration, and exten-sive clinical experience with these drugs will make such clinical studies very feasible.

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