Anti-CD3 antibodies modulate anti-factor VIII immune responses in hemophilia A mice after factor VIII plasmid-mediated gene therapy

Running title: Anti-CD3 modulates anti-factor VIII immune responses

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ABSTRACT

One of the major obstacles in gene therapy is the generation of immune responses directed against transgene product. Five consecutive anti-CD3 treatments concomitant with factor VIII (FVIII) plasmid injection prevented the formation of inhibitory antibodies against FVIII and achieved persistent, therapeutic-level of FVIII gene expression in treated hemophilia A mice. Repeated plasmid gene transfer is applicable in tolerized mice without eliciting immune responses. Anti-CD3 treatment significantly depleted both CD4+ and CD8+ T cells in treated mice, while increased TGF-β levels in plasma and the frequency of both CD4+CD25+FoxP3+ and CD4+CD25-Foxp3+ regulatory T cells (Tregs) in the initial few weeks post treatment. Although prior depletion of CD4+CD25+ cells did not abrogate tolerance induction, adoptive transfer of CD4+ cells from tolerized mice at 6 weeks post treatment protected recipient mice from anti-FVIII immune responses. Anti-CD3 treated mice mounted immune responses against both T-dependent and T-independent neo-antigens indicating anti-CD3 did not hamper the immune systems for long-term. Concomitant FVIII plasmid+anti-CD3 treatment induced long-term tolerance specific to FVIII via a mechanism involving the increase in TGF-β levels and the generation of adaptive FVIII-specific CD4+Foxp3+Tregs at the periphery. Furthermore, anti-CD3 can reduce the titers of pre-existing anti-FVIII inhibitory antibodies in hemophilia A mice.
INTRODUCTION

Gene therapy protocols using viral and nonviral vectors have been devised for many diseases and employed in preclinical studies. Recent studies indicate that immune responses against vectors or transgene products can become major obstacles for successful application of gene therapy\textsuperscript{1-3}.

Hemophilia A, a congenital bleeding disorder caused by a monogenic defect of coagulation factor VIII (FVIII), is an ideal candidate for the successful application of somatic cell gene therapy. Previous pre-clinical and clinical trials demonstrated that viral and nonviral gene transfer of FVIII\textsuperscript{4-9} often results in transient gene expression in the absence of immunosuppression. It is therefore essential to develop safe and effective methods to modulate immune responses against transgene products, vectors, and/or gene-engineered cells to ensure the success of gene therapy.

Therapeutic biological compounds have been used successfully to modulate the immune system. The development of the first humanized anti-CD3 monoclonal antibody (OKT3) spurred a spate of studies with CD3-specific antibodies for the treatment of immune-mediated diseases\textsuperscript{10-15}. Clinical trials with this monoclonal antibody included treatment of acute renal allograft rejection\textsuperscript{16, 17}, autoimmune insulin-dependent diabetes\textsuperscript{18}, and psoriatic arthritis\textsuperscript{19}, and prevention of islet allograft rejection\textsuperscript{20}. The results of these trials are encouraging and anti-CD3 treatment did not elicit major side effects\textsuperscript{16, 17}. Anti-CD3 therapy was demonstrated to promote, in both transplantation\textsuperscript{21, 22} and autoimmune settings\textsuperscript{23}, antigen-specific immune tolerance. In patients with recent onset autoimmune diabetes, reversion of hyperglycemia was achieved by combination therapy of anti-CD3 injections and intranasal application of proinsulin peptide, but not by either agent alone\textsuperscript{23}. 


It was suggested that the combination therapy induces large numbers of islet cell-specific Tregs, whereas anti-CD3 or proinsulin alone were insufficient to prime Tregs.

Experimental evidence supports the hypothesis that there are two subsets of Tregs which differ in specificity and effector mechanism\textsuperscript{24}. Natural Tregs emerge from the thymus as a distinct lineage, whereas adaptive Tregs are induced at the periphery from CD4\textsuperscript{+}CD25\textsuperscript{−} T cells under specific conditions, i.e. antigenic stimulation in the presence of a particular cytokine environment or altered TCR signal transduction. Studies in mice engineered to express a Foxp3 reporter confirmed that Foxp3 is a lineage marker of Tregs and correlates with suppressor activity irrespective of CD25 expression\textsuperscript{25-27}. In particular, in an autoimmune nonobese diabetic (NOD) mouse model, anti-CD3 treatment induced adaptive CD4\textsuperscript{+}CD25\textsuperscript{low}Foxp3\textsuperscript{+}Tregs in the periphery that suppress T cell immunity in a TGF-β dependent manner\textsuperscript{28}.

Here we report the successful use of the anti-CD3 monoclonal antibody in modulating immune responses against FVIII after gene therapy. Five consecutive injections of anti-CD3 induced tolerance to FVIII in hemophilia A mice, manifested by persistence of FVIII activity and the absence of circulatory FVIII-specific inhibitory antibodies. The treatment with anti-CD3 caused temporary depletion of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and increased frequency of CD4\textsuperscript{+}Foxp3\textsuperscript{+}Tregs. Long-term tolerance was confirmed by a second plasmid challenge to anti-CD3 tolerized mice without eliciting FVIII-specific immune responses. In addition, anti-CD3 antibody treatment did not damper immune response toward unrelated antigens after treated mice recovered from transient immunosuppression.
MATERIALS AND METHODS

Mice. All mice were kept in accordance with National Institutes of Health guidelines for animal care and the guideline of Seattle Children’s Research Institute, and maintained at a specific pathogen-free facility. The animal protocols used in this study were approved by the institutional IACUC Committee of Seattle Children’s Research Institute. Hemophilia A mice in a 129/SV × C57BL/6 mixed genetic background were generated by targeted disruption of exon 16 of the FVIII gene and bred in our animal facility.

Antibodies. Anti-mouse-CD3ε monoclonal antibodies (145-2C11), anti-mouse-CD25 monoclonal antibodies (PC61), and mouse IgG1 isotype control were purchased from BioXCell (West Lebanon, NH). Anti-mouse-Foxp3 (FJK-16s)-FITC, anti-mouse-CD25 (PC61)-APC, anti-mouse-CD25 (7D4)-APC, anti-mouse-CD4 (L3T4)-PE and anti-mouse-CD8 (Ly3)-APC were purchased from eBioscience (San Diego, CA). Anti-CD4 (L3T4)-Alexa Flour® 700 (BD) was purchased from BD Pharmingen (San Jose, CA).

Gene Transfer of FVIII into hemophilia A mice with immunomodulation regimen by anti-CD3 antibodies. Hemophilia A mice were injected with 50 µg FVIII plasmid (pBS-HCRHP-FVIIIA) in 2 ml phosphate-buffered saline (PBS) via tail vein in 8-10 seconds. For immunomodulation, FVIII plasmid treated mice were given intravenous (i.v.) injections of anti-CD3 antibody at a dose of 40 µg at the time of plasmid injection and subsequent daily injections for four additional days. Groups of anti-CD3 only treated mice, FVIII plasmid only treated mice and naïve mice were included as controls. Blood samples were taken from the retro-orbital plexus at serial time points and assessed for FVIII activity and anti-FVIII antibody levels. Selected immunomodulated mice received
a second plasmid challenge at 23 weeks after the first plasmid injection, or received 2.5 units (approximately 500 ng) recombinant human FVIII (Baxter, Westlake Village, CA) emulsified in CFA (Sigma) at 12 weeks after the first plasmid injection.

Assays for measuring FVIII activities and anti-FVIII inhibitory antibodies. FVIII activity was measured by a modified clotting assay using reagents to measure activated partial thromboplastin time (APTT) and FVIII deficient plasma\textsuperscript{9, 30}. FVIII activity was calculated from a standard curve generated by using serially diluted normal human pooled plasma.

Inhibitory antibodies against FVIII were measured by Bethesda assay as previously described\textsuperscript{31}. hFVIII specific antibodies (total IgGs) were assessed by ELISA using the same method described previously\textsuperscript{9}. Sera collected 2 weeks before and after second FVIII plasmid challenge were used.

\textit{T-cell staining and flow Cytometry analysis.} Single-cell suspensions were prepared by mechanical disruption of lymph nodes (LN) (superficial cervical) and spleens of FVIII plasmid+anti-CD3, FVIII plasmid only, anti-CD3 only or untreated naïve control mice. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood after lysis of red blood cells. Cells were first stained for surface markers CD3, CD4, CD8, and CD25, and subsequently stained intracellularly for Foxp3 following the company protocol (eBioscience). Flow cytometry analysis was performed using a FACS LSRII flow cytometer (Becton Dickinson, Palo Alto, CA) and analyzed using FlowJo software (Treestar, Ashland, OR)

\textit{Proliferation assay and suppressive assay.} CD4\textsuperscript{+} T cells were isolated from spleens of mice by magnetic activated cells sorting (MACS) (Miltenyi Biotec Inc, Auburn, CA).
The CD4⁺CD25⁻, CD4⁺CD25⁺ subsets were further isolated from the CD4⁺ T cells using a CD25⁺ Treg MACS isolation kit (Miltenyi Biotec Inc). For proliferation, 1.0x10⁵ CD4⁺ T cells were co-cultured with 1.0x10⁵ CD4⁻ cells (irradiated, used as antigen presenting cells, APC) per well in 96-well round-bottom plate with or without the presence of FVIII at 10U/ml (1U=100ng FVIII protein) for 72h, followed by adding 1µCi [³H]thymidine per well for the final 18 hour. [³H]thymidine incorporation was measured as counts per minute (c.p.m.) in a Betaplate scintillation counter (Perkin-Elmer). For suppressive assay, CD4⁺T cells from spleens of FVIII plasmid only treated mice were used as responders and CD4⁺CD25⁺T cells from spleens of FVIII plasmid+anti-CD3 treated mice at week two or week six after plasmid injection or from spleens of age matched naïve mice were used as Tregs from treated or control mice. To the co-culture of 0.8x10⁵ CD4⁺ T cells and 1.5x10⁵ APCs, we added CD4⁺CD25⁺T cells at indicated ratios. The cultures were stimulated with 10U/ml of FVIII for 72h, followed by adding 1µCi [³H]thymidine per well for the final 18 hours. All cultures were done in triplicates. Suppression was calculated as:

\[
\% \text{ suppression} = (1 - \frac{\text{c.p.m. } \text{CD4}^+ \text{CD25}^- \text{T cells} + \text{CD4}^+ \text{CD25}^+ \text{T cells}}{\text{c.p.m. CD4}^+ \text{CD25}^- \text{T cells alone}}) \times 100\%
\]

Adoptive Transfer of CD4⁺ T cells into Syngeneic hemophilia A Mice. CD4⁺T cells were isolated as described above from spleens of FVIII plasmid+anti-CD3, FVIII plasmid only and naïve mice. 2-4x10⁶ CD4⁺T cells suspended in 300 µl PBS were injected into syngeneic mice via tail vein. Mice were subsequently challenged by hydrodynamic delivery of FVIII plasmid the following day. FVIII activity and anti-FVIII inhibitory antibody levels were monitored over time.

Tregs depletion by anti-CD25 monoclonal antibodies. Hemophilia A mice (n=4) received
a single injection of 1mg anti-CD25 monoclonal antibodies (PC61) at 3 days prior to the first anti-CD3 and FVIII plasmid co-injection. Two weeks later, each mouse received an additional injection of 0.5mg anti-CD25. Depletion of CD4⁺CD25⁺ cells was examined by flow cytometry assay of blood samples obtained from treated mice using the 7D4 clone of anti-CD25. In an independent experiment, hemophilia A mice (n=4), co-injected with FVIII plasmid and anti-CD3 as described above, were treated with 0.5mg anti-CD25 every week for four weeks. FVIII activities and anti-FVIII inhibitory antibodies in those mice were monitored over time.

**Quantitation of systemic TGF-β levels.** To quantitate systemic TGF-β1 in mice, plasma was pre-treated with 1 N HCL, then assayed using a TGF-β1 ELISA kit (BD Bioscience, San Jose, CA) according to the manufacturer’s recommendation and the data interpolated against the linear range on the standard curves.

**Immunization with the neo-T-independent antigen TNP-ficoll.** Groups of tolerized mice (n=4) and FVIII plasmid only treated mice (n=3) were immunized with 20 µg TNP-ficoll intraperitoneally each 24 weeks after plasmid injection. Serum was collected 10 days after TNP-ficoll injection. Anti-TNP IgG3 and anti-TNP IgM in serum were detected by ELISA.

**Immunization of mice with bacteriophage Φx174.** Bacteriophage Φx174 was prepared as previously described. The stock solution of 1x10¹¹ plaque-forming units (PFUs) per ml was diluted and injected intraperitoneally into two tolerized hemophilia A mice 6 months after plasmid injection and two naive hemophilia A mice at a dose of 1x10¹⁰ PFU/kg (2x10⁸ PFU/mouse). A secondary immunization was carried out 4 weeks after the primary immunization.
Serum samples were analyzed for phage-neutralizing antibody activity and expressed as the rate of phage inactivation (Kv) using the standard formula \(^{33}\): 
\[
Kv = \left( \frac{\text{dilution of serum}}{\text{time of phage-serum incubation in minutes}} \right) \times \ln \left( \frac{\text{phage assay PFU at 0 minute}}{\text{phage assay PFU at 60 minutes}} \right).
\]
Antibody resistant to 2 mercaptoethanol (2-ME) was considered to be of the IgG isotype.

*Statistics.* Data are expressed as mean ± S.D. The statistical significance of the difference between means was determined using the 2-tailed Student’s *t* test. Differences were considered significant at P<0.05.
RESULTS

Anti-CD3 induces tolerance to FVIII after FVIII plasmid-mediated gene therapy

To test if CD3-specific antibody can modulate transgene-specific immune responses following FVIII plasmid-mediated gene therapy, we injected hemophilia A mice (n=13/group) with 50 µg FVIII plasmid by hydrodynamic injection (day 0) and gave five daily infusions of anti-CD3ε at a dose of 40µg/day (days 0-4) via tail vein. Plasma samples were collected from treated and naïve mice at scheduled time points and FVIII activities and inhibitory antibody titers were assessed.

As we have previously shown\textsuperscript{34, 35}, injection of FVIII plasmid alone produced short-term high-levels of FVIII activity in hemophilia A mice, followed by a gradual decrease to undetectable levels in 2-4 weeks due to the development of anti-FVIII inhibitory antibodies (Fig. 1a&b). In contrast, immuno-modulation with anti-CD3 led to persistently therapeutic levels of FVIII activities (30-105% of FVIII levels in normal human plasma) for up to 24 weeks in 11 out of the 13 FVIII plasmid treated mice (Fig. 1c). For the remaining two anti-CD3 treated mice, FVIII activities persisted at therapeutic levels for 10 and 15 weeks, respectively, before dropping to undetectable levels (Fig 1c). Most importantly, none of the 13 treated mice developed FVIII inhibitory antibodies throughout the 24 weeks experimental period (Fig. 1d). In addition, when we treated mice using a lower dosage of anti-CD3 (5µg/day at days 0-4, n=4/group), none of the mice developed antibody response up to 12 weeks past plasmid injection, indicating that this lower dosage was also able to induce tolerance to FVIII in hemophilia A mice.

To assess transgene-specific T cell proliferation, we isolated splenic CD4\textsuperscript{+}T cells from naïve, anti-CD3 only, FVIII plasmid only and FVIII plasmid+anti-CD3 treated mice
3 weeks after plasmid injection. These responder cells were co-cultured with irradiated splenic CD4+ cells from a naïve mouse which served as APCs. Without stimulant, T cells from none of the experimental groups proliferated (Fig. 2). When FVIII protein was added at optimal concentration, CD4+T cells from FVIII plasmid only treated group proliferated robustly, as expected. In contrast, CD4+T cells from FVIII plasmid+anti-CD3 treated mice 3 weeks (Figure 2) and 6 weeks (data not shown) after plasmid injection showed only minor non-specific proliferation, similar to CD4+T cells from control naive and anti-CD3 only treated mice. These results are consistent with the observations that anti-CD3 treatment effectively suppressed transgene-specific immune responses following gene transfer of FVIII plasmid.

To further investigate whether anti-CD3 immunomodulation induced short term unresponsiveness or long term tolerance to FVIII, we challenge the anti-CD3 immunomodulated mice a second time at 23 weeks after the 1st FVIII plasmid injection. For each independent experiment (n=3 mice/group, total 2 groups, representative data from 1 group were shown In Fig. 3), we chose 2 mice in which long term FVIII expression had been established and 1 mouse in which FVIII expression fell to undetectable levels within 12 weeks after treatment. The 2nd challenge with FVIII plasmid induced a short burst of high-level FVIII activities in all three mice (Fig. 3a). Mice with persistent FVIII expression after the 1st FVIII plasmid injection continued to generate detectable FVIII expression after the 2nd challenge (Fig. 3a). In contrast, the mouse with undetectable FVIII expression 12 weeks after the 1st treatment only boosted FVIII expression for a couple of weeks following the 2nd plasmid challenge then became again undetectable (Fig. 3a). None of the anti-CD3 treated mice developed detectable
inhibitory antibodies against FVIII after the 2nd challenge (Fig. 3b). In an independent control experiment, we challenged FVIII plasmid only treated mice with a 2nd FVIII plasmid injection at 8 weeks. In contrast to mice treated with anti-CD3, none of the FVIII plasmid only treated mice produced a boost in FVIII activity but all showed a significant increase in anti-FVIII antibody after 2nd challenge.

Next, we assessed the total human FVIII specific antibodies (total IgGs) in FVIII plasmid only mice, FVIII plasmid + anti-CD3 mice of short-term FVIII expression or long-term expression (n=2/group) before and after 2nd plasmid challenge. We detected high levels of FVIII specific antibodies (~27μg/ml) in FVIII plasmid only mice but not in FVIII plasmid + anti-CD3 mice of long-term FVIII expression before and after 2nd plasmid challenge (Supplement Table 1). However, we detected low levels of non-neutralizing antibodies (~70ng/ml) in FVIII plasmid + anti-CD3 mice of short-term FVIII expression at 2 weeks before and after 2nd plasmid injection (Supplement Table 1) which may be responsible for eliminating circulating FVIII protein in mouse plasma.

Furthermore, we challenged two groups of mice (FVIII plasmid only and FVIII plasmid+anti-CD3 tolerized) with 2.5 units of recombinant hFVIII emulsified in CFA at 12 weeks after FVIII plasmid injection. Two weeks after the second challenge, we found that FVIII expression persisted at the same level as that before the challenge, and no inhibitor antibody response was detected in previously FVIII plasmid+anti-CD3 tolerized mice (data not shown). In contrast, CFA+FVIII protein challenge induced a robust secondary immune response in FVIII plasmid only treated mice. FVIII plasmid+anti-CD3 treated mice were tolerant to hFVIII protein even when presented in stringent conditions. Taken together, these data indicate that anti-CD3 treatment exerted long-term
partial or complete protection against FVIII-specific immune responses following FVIII plasmid-mediated gene therapy in hemophilia A mice.

**Anti-CD3 treatment induces depletion of CD4+ and CD8+ T cells and an increase in the frequency of CD4+Foxp3+ T cell**

Since anti-CD3 treatment targets the TCR complex, we investigated the T cell compartments of mice injected with anti-CD3. We analyzed spleen, lymph node and peripheral blood in groups of mice (n=3/group) treated with anti-CD3 only, FVIII plasmid only, or FVIII plasmid+anti-CD3 and naïve control mice. On day 3 after the last of five anti-CD3 injections (day 8 after FVIII plasmid injection) approximately 80-90% of CD4+ and CD8+ T cells were depleted in spleen, lymph nodes and blood of anti-CD3 only and FVIII plasmid+anti-CD3 treated mice but not in plasmid only treated animals (Fig. 4a and 4d). A detailed flow cytometric analysis of peripheral blood lymphocytes showed that the levels of CD4+T cells declined most significantly at week 1 and gradually returned to normal within 8 weeks in both anti-CD3 only and FVIII plasmid+anti-CD3 treated groups (Fig. 4d). FVIII plasmid injection had no effect on anti-CD3 induced CD4+T cell depletion.

Anti-CD3 treatment resulted in significantly higher percentage of CD4+Foxp3+T cells in spleen (Fig. 4a), lymph node (supplement Fig. 1), and peripheral blood (Fig. 4e) in mice. The absolute number of splenic CD4+Foxp3+ T cells in FVIII plasmid+anti-CD3 treated mice were approximately 90% of that observed in FVIII plasmid only treated mice based on total number of spleen cells and flow analysis (n=3, p=0.37)(Table 1). Consistent with this observation is our finding that CD3 expression is higher on the surface of splenic CD4+Foxp3+T cells (median fluorescence intensity (MFI)=6147±217,
Fig. 4b, left panel, dark line) than on splenic CD4⁺Foxp3⁻T cells (MFI= 4086±42, Fig. 4b, left panel, thin line) in plasmid FVIII only treated mice. With anti-CD3 treatment, the CD3high subsets in both populations of CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ were depleted (Fig. 4b, right panel). Anti-CD3 treatment selectively depleted more CD4⁺Foxp3⁻ T cells than CD4⁺Foxp3⁺ T cells.

Furthermore, following anti-CD3 treatment, the frequency of CD4⁺CD25⁺Foxp3⁺ T cells increased significantly in the peripheral blood with or without concomitant FVIII plasmid injection (Fig. 4f). Although there was no difference in the percentage of CD4⁺CD25⁺Foxp3⁺T cells between mice treated with anti-CD3 only and FVIII plasmid+anti-CD3, there was a significant difference in the percentage of CD4⁺Foxp3⁺ T cells between these two groups of mice (Fig. 4e, p<0.05), indicating that antigen exposure promoted generation of CD4⁺Foxp3⁺ T cells. Similar results were observed in the CD4⁺ T cell compartments of spleen (Fig. 4a) and lymph node (Supplement Fig. 1). Despite the percentage of CD25⁺Foxp3⁺Tregs in CD4⁺T cells increased significantly, the absolute number of splenic CD4⁺CD25⁺Foxp3⁺Tregs in FVIII plasmid+anti-CD3 treated mice were approximately 67% of that observed in FVIII plasmid only treated mice at day 3 (n=3, p<0.05)(Table 1).

A close examination of CD25 expression on CD4⁺Foxp3⁺T cells revealed that about half of the CD4⁺Foxp3⁺ T cells is CD25⁻ and only a small fraction of CD4⁺Foxp3⁺ T cells is CD25hi in spleens (Fig. 4C) and lymph nodes (Supplement Fig. 1) of mice treated with FVIII plasmid+anti-CD3 at day 8 after FVIII plasmid injection. In comparison, about half of the CD4⁺Foxp3⁺T cells is CD25hi and only a small fraction of the CD4⁺Foxp3⁺ T cells is CD25⁻ in spleens and lymph nodes of naïve mice and FVIII plasmid only treated mice.
(Fig. 4c and supplement Fig. 1). At 4 and 6 weeks after plasmid injection, the distribution of CD25+ and CD25− cells in CD4+Foxp3+T cells in spleens of FVIII plasmid+anti-CD3 mice gradually returned to levels comparable to that in spleens of naïve mice and FVIII plasmid only treated mice (Fig. 4c).

A recent study suggested that CD3 specific antibody increases the level of transforming growth factor-β1 (TGF-β1) in vivo, leading to the generation of CD4+CD25+Foxp3+ regulatory T cells and tolerance \(^3^6\). We investigated the TGF-β1 levels in the plasma of naïve and FVIII plasmid+anti-CD3 treated mice. Two FVIII plasmid+anti-CD3 tolerized mice (mouse #4 and #5, Table 2) had increased TGF-β1 levels at day 3 and/or day 10 after the last anti-CD3 injection, compared to that of naive mice (mouse #1, #2, and #3). Mouse #6 which lost FVIII activity at about 12 weeks did not produce higher plasma TGF-β1 at day 3 and/or day 10.

**Examination of the role of CD4+CD25+Foxp3+ Tregs in tolerance induction by anti-CD3 treatment**

Since Tregs have been involved in the induction of peripheral tolerance in many settings, we tested a possible role of Tregs in our model. We first examined the suppressive activity of CD4+CD25+ T cells isolated from FVIII plasmid+anti-CD3 tolerized and from naïve mice at two weeks following FVIII plasmid injection in a FVIII-specific suppression assay. CD4+T cells from FVIII plasmid only treated mice were used as responder T cells. CD4+CD25+Tregs of naïve mice showed no suppression on FVIII-specific proliferation. Surprisingly, CD4+CD25+Tregs of FVIII plasmid+anti-CD3 mice, collected two weeks following FVIII plasmid injection did not suppress FVIII specific proliferation (Fig. 5a). However, when we used CD4+CD25+Treg cells isolated from
tolerized mice six weeks or more after FVIII plasmid injection, we observed FVIII-specific suppression (Fig. 5b).

Next, we performed adoptive transfer experiments. Pooled CD4^+T cells were isolated from the spleens of naïve mice, *FVIII* plasmid only and *FVIII* plasmid+anti-CD3 treated mice 2 or 6 weeks after plasmid injection. 2-4x10^6 of CD4^+ cells were transferred into naïve syngeneic hemophilia A mice. The recipient mice were challenged with *FVIII* plasmid one day after cell transfer, and FVIII expression levels and inhibitory antibodies production were monitored. As expected, transfer of CD4^+T cells of naïve mice didn’t affect FVIII expression and formation of FVIII inhibitors in recipient mice after *FVIII* plasmid challenge (Table 3). Transfer of CD4^+T cells obtained from *FVIII* plasmid only treated mice 2 weeks after plasmid injection accelerated the loss of FVIII activity and the formation of inhibitory antibodies in recipient mice. Interestingly, transfer of CD4^+T cells derived from *FVIII* plasmid+anti-CD3 treated mice “2” weeks after plasmid injection neither affect the expression of FVIII nor the formation of FVIII inhibitors in recipient mice, whereas transfer of CD4^+T cells derived from *FVIII* plasmid+anti-CD3 treated mice “6” weeks after plasmid injection prolonged the expression of FVIII and delayed the formation of FVIII inhibitors in recipient mice, compared with transfer of CD4^+T cells derived from naïve mice.

To deplete CD4^+CD25^+Tregs, we treated hemophilia A mice with anti-CD25 (clone: PC61) monoclonal antibodies prior to *FVIII* plasmid and anti-CD3 injection as outlined in Fig. 5a. Injection of 1mg anti-CD25 at day 0 plus a second injection of 0.5mg anti-CD25 at day 14 (Fig. 6a) into hemophilia A mice depleted 99% of CD4^+CD25^+T cells (including both CD4^+CD25^+Foxp3^+ and CD4^+CD25^+Foxp3^-T cells) for up to four
weeks, however, did not affect CD4+CD25-Foxp3+T cells (Fig. 6b & c). The percentage of CD4+CD25-Foxp3+ T cells in anti-CD25+FVIII plasmid+anti-CD3 treated mice was significantly higher than that observed in naïve mice (P<0.05) (Fig. 6b&c). After anti-CD25+FVIII plasmid+anti-CD3 treatment, 2 out of 4 mice lost FVIII expression at 8 to 12 weeks after plasmid injection, whereas the other 2 mice achieved persistent FVIII expression for 112 days (experimental duration). Similar results (2 of 4 mice lost expression) were observed in the control IgG1+FVIII plasmid+anti-CD3 treated mice. None of the mice developed inhibitory antibodies during the time of study (data not shown). These results are in agreement with a previous report\textsuperscript{14}, suggesting that prior treatment with anti-CD25 does not abrogate anti-CD3 induced tolerance.

**Un-related T-dependent and T-independent antigen challenge**

Since anti-CD3 treatment temporarily depleted a large portion of the T cell compartment, the long term effect on the immune system was investigated. Two FVIII plasmid+anti-CD3 tolerized mice and two control naïve mice were inoculated with the T-dependent antigen, bacteriophage Φx174\textsuperscript{33}, and another two groups with the T-independent antigen, TNP-ficoll 24 weeks after plasmid delivery. Peak antibody titers observed at 2 weeks post primary and 1 week post secondary Φx174 immunization and isotype switching (percent phage specific IgG) during secondary immunization were not different between the 2 groups (Fig. 7a). As shown in Fig. 7, no significant difference in serum levels of anti-TNP IgG3 (Fig. 7b) or anti-TNP IgM (Fig. 7c) were found between tolerized mice and FVIII plasmid only treated mice. Together, these data indicated that transient immunomodulation with anti-CD3 does not adversely affect immune responses to T-dependent or T-independent neoantigens.
Anti-CD3 can reduce the titers of pre-existing inhibitory antibodies in hemophilia A mice.

To date, there is no clinical or laboratory risk factors that allow the identification of a patient who will develop antibody. Therefore, it is very important to develop a strategy to treat pre-existing inhibitory antibodies. Thus, we injected a group of hemophilia A mice (n=4/group) with 50 µg $FVIII$ plasmid by hydrodynamic injection (week -8). Eight weeks post plasmid injection, 2 mice developed high-titer anti-FVIII antibody and 2 mice developed low-titer antibody. All 4 mice were subjected to five daily infusions of anti-CD3ε at a dose of 40µg/day (days 0-4) via tail vein. Anti-FVIII antibody titers were significantly reduced or eliminated and FVIII activities recovered to detectable levels 2 weeks following anti-CD3 treatment (Table 4). However, FVIII activities declined and anti-FVIII antibody titers re-appeared at low-titers or increased at later time points. These data indicated that anti-CD3 can partially modulate pre-existing anti-FVIII immune responses.
DISCUSSION

Previously reported data have identified the prominent immune response against FVIII as a major obstacle for successful gene therapy for hemophilia A, whether the FVIII gene was delivered by viral vectors\textsuperscript{4-7} or naked plasmid DNA\textsuperscript{9}. Some of the existing strategies, such as using cell-type specific promoters, can only reduce but not eliminate the immune responses\textsuperscript{9, 30}. Here we report a promising strategy to modulate anti-FVIII responses in hemophilia A mice after gene therapy by immunomodulation with anti-CD3 antibody.

Five consecutive injections of modest doses of anti-CD3 delivered concomitantly with plasmid DNA encoding the FVIII gene resulted in long-term, stable FVIII expression in 85\% of the treated hemophilia A mice, while none developed detectable neutralizing antibodies (inhibitors of FVIII). In comparison, FVIII expression in FVIII plasmid only treated mice declined to background levels in 2-4 weeks and inhibitors of FVIII could be detected as early as 3-4 weeks\textsuperscript{34, 35}. Splenic T cells isolated from FVIII plasmid+anti-CD3 treated mice showed no indication of recall proliferation to FVIII stimulation \textit{in vitro}. Furthermore, with a 2\textsuperscript{nd} plasmid challenge at week 23, when mice had long recovered from the immune suppression of anti-CD3, the treated mice failed to elicit an immune response. Moreover, FVIII challenge under stringent condition (emulsified in CFA) could not break the induced tolerance in FVIII plasmid+anti-CD3 mice. The two mice that lost FVIII expression between week 10 and 15 have had low levels non-neutralizing anti-FVIII antibodies (supplement Table 1) that may have led to the eventual loss of FVIII activity. Taken together, these data indicate that anti-CD3 immunomodulation following FVIII plasmid-mediated gene therapy induced partial or
complete long-term protection against FVIII-specific immune responses in hemophilia A mice.

As expected, anti-CD3 treatment resulted in depletion of approximately 80-90% of CD4+ and CD8+ T cells in spleen and lymph nodes and blood at day 8 after FVIII plasmid injection. This loss of T cells was accompanied by a significant increase in the percentage of CD4+Foxp3+ T cells. However, the absolute number of CD4+Foxp3+T cells in FVIII plasmid+anti-CD3 mice were comparable to that in FVIII plasmid only mice. The increases in frequency of CD4+Foxp3+T cells at this early time points could come from two sources: (1) anti-CD3 selectively depletes more of the CD4+Foxp3− population within the CD4+ T cells, (2) anti-CD3 promotes generation of new FVIII-specific CD4+Foxp3+ T cells in the periphery.

The CD4+Foxp3+T cell population contains CD25+ (including both CD25hi and CD25low T cells) and CD25− T cells. Interestingly, as shown in the results section, although the percentage of CD4+CD25+Foxp3+T cells increased following anti-CD3 treatment, the absolute numbers slightly decreased. This is most likely due to the selective depletion of activated effector and naive T cells in the CD4 compartment due to higher CD3 expression in these populations (Fig. 4b), leading to the enrichment of natural, thymus-originated CD4+CD25+Foxp3−Tregs. On the other hand, both the percentage and absolute numbers of CD4+CD25−Foxp3+ cells increased significantly in the spleens of FVIII plasmid+anti-CD3 treated mice compared to FVIII plasmid only treated and naive mice (Table 1). These data indicates that anti-CD3 induced generation of new CD4+CD25−Foxp3+ cells at the early time points following anti-CD3 treatment.
Our data indicated that at the early time point up to 2 weeks post plasmid injection, the CD4⁺CD25⁺Foxp3⁺ Tregs contain mostly natural, thymus-originated Tregs which had little contribution to FVIII-specific tolerance induction. This was concluded by three separate experiments: (1) CD4⁺CD25⁺T cells isolated from MACS (including CD25<sup>hi</sup> and CD25<sup>low</sup>, >80% Foxp3⁺) from spleens of FVIII plasmid+anti-CD3 treated mice at week 2 after plasmid injection could not suppress proliferation of FVIII-specific effector T cells (Fig. 5a), (2) These CD4⁺CD25⁺T cells could not transfer dominant, FVIII-specific tolerance to syngenic naive mice (data not shown), (3) Depletion of CD4⁺CD25⁺T cells by i.v. injection of anti-CD25 antibodies could not abrogate FVIII-specific tolerance induced by FVIII plasmid+ ani-CD3 (Fig. 6). Nevertheless, the number of mice in the anti-CD25 group is not large enough to firmly conclude the anti-CD25 antibody does not abrogate anti-CD3 mediated tolerance.

We hypothesize that the newly generated CD4⁺CD25⁻Foxp3⁺ T cells are the Tregs responsible for tolerance induction at the initial stage of FVIII plasmid+anti-CD3 treatment. Studies in mice engineered to express a Foxp3 reporter demonstrated that Foxp3 expression is confined to a subset of αβ T cells and correlates with suppressive activity irrespective of CD25 expression<sup>25-27</sup>. In addition, a recent study conducted with SH2 domain-containing inositol 5-phosphatase (SHIP) deficient mice shows that CD25⁺ T cells express higher Foxp3 levels, and CD4⁺CD25⁻Foxp3⁺ T cells have profound immunosuppressive capacity in vitro and in vivo<sup>37</sup>. Moreover, there is now compelling evidence that “adaptive” Tregs may be generated from peripheral CD4⁺CD25⁻Foxp3⁻ T cells under well defined conditions (i.e. the type of antigen stimulation, the nature of the APCs, and cytokine milieu)<sup>28, 32, 36, 38</sup>. Furthermore, we found that the numbers of
CD4⁺CD25⁻Foxp3⁺ T cells increased, whereas comparable numbers of CD4⁺CD25⁻ Foxp3⁺ T cells decreased in spleens of FVIII plasmid+anti-CD3 treated mice from day 8 to week 4 and 6 after FVIII plasmid injection (Table 1), implying conversion of CD25⁻ cells to CD25⁺ cells within the CD4⁺Foxp3⁺ population. The induced CD4⁺CD25⁺Foxp3⁺ cells are demonstrated to suppress FVIII-specific immune responses at later time points: (1) CD4⁺CD25⁺T cells from spleens of FVIII plasmid+anti-CD3 treated mice at week 6 after plasmid injection suppressed proliferation of FVIII-specific effector T cells (Fig. 6b), (2) The CD4⁺T cells transferred dominant, FVIII-specific tolerance to syngenic naive mice (Table 3).

It has been shown that CD3 specific antibody treatment promotes the production of systemic transforming growth factor-β1 (TGF-β1), which is known to induce CD4⁺Foxp3⁺ regulatory T cells28, 32, 36, 38. Chatenoud et al. reported that administration of a neutralizing anti-TGF-β1 antibody abrogated remission of diabetes induced by anti-CD3 antibody32, indicating the importance of TGF-β1 in maintaining tolerance. In our study, anti-CD3 treated mice exhibited increased TGF-β1 levels in the blood during a period of 8 days following treatment in tolerized mice but not in mice with inhibitors. All together, we hypothesize that FVIII plasmid+anti-CD3 treatment induced TGF-β1 dependent conversion of CD4⁺CD25⁻ cells to “adaptive” CD4⁺CD25⁺Foxp3⁺Tregs at the periphery at early time point after the treatment; “adaptive” CD4⁺CD25⁺Foxp3⁺Tregs eventually matured into CD4⁺CD25⁺Foxp3⁺Tregs to maintain long term FVIII-specific tolerance. However, we can not rule out participation of other types of regulatory T cells such Tr1 in inducing tolerance in our experimental setting.
Waters et al. recently reported that anti-CD3 is also able to induce tolerance to FVIII following repeated injections of FVIII proteins in hemophilia A mice via a CD4+CD25+Treg cell dependent mechanism\textsuperscript{39}. Their result is also consistent with our study demonstrating that Tregs are pivotal for establishing FVIII tolerance. However, the development and characteristics of functional antigen-specific Treg cells are different in these two studies. We propose that inducible CD4+CD25\textsuperscript{low}Foxp3\textsuperscript{+}Tregs initiated the tolerance and then matured into CD4+CD25\textsuperscript{+}Foxp3\textsuperscript{+}Tregs for maintaining long term tolerance. In their model, CD4+CD25\textsuperscript{+}Foxp3\textsuperscript{+}Tregs are important for both induction and maintenance of the tolerance. This difference may be due to the different protocols of anti-CD3 treatment. Waters et al. injected anti-CD3 prior to FVIII protein challenge in which natural CD4+CD25\textsuperscript{+}Foxp3\textsuperscript{+}Tregs enriched by anti-CD3 treatment may convert to FVIII specific CD4+CD25\textsuperscript{+}Foxp3\textsuperscript{+}Tregs upon encountering FVIII later, thus depletion of CD4+CD25\textsuperscript{+} cells by anti-CD25 abrogated the tolerance induction by anti-CD3 treatment. In our study, we injected anti-CD3 with FVIII plasmid concomitantly, therefore antigen specific CD4+CD25\textsuperscript{low}Foxp3\textsuperscript{+}Tregs were induced at early time point and the tolerance was not abrogated by anti-CD25 treatment. Additionally, Waters et al. used a non-FcR binding, F(ab')\textsubscript{2} form of anti-CD3 and we used a complete form of anti-CD3. Finally, our study demonstrated that in majority of the treated mice, FVIII plasmid+anti-CD3 treatment can induce long-term tolerance which protected the mice from antibody production following the second challenge with either FVIII plasmid or FVIII protein+CFA. Waters et al. did not perform the second challenge experiment therefore it is uncertain if their protocol of pre-treatment with anti-CD3 established the long-term tolerance.
In summary, concomitant immunomodulation by anti-CD3 with gene transfer of \textit{FVIII} plasmid achieved long term FVIII expression in hemophilia A mice which was not affected by repeated plasmid DNA applications. FVIII expression greater than 30\% of normal human plasma lasted for 34 weeks after gene delivery without detectable anti-FVIII immune responses. Furthermore, anti-CD3 treatment did not affect the host to mount immune responses to T-dependent and T-independent neo-antigens. Anti-human CD3 has been approved by FDA for clinical use and has been widely applied in transplantation and autoimmune models. The dosage and schedule used in our study are comparable to those used in human trials\textsuperscript{14, 15, 18, 23}. Anti-CD3 immunomodulation has the potential to be a safe and effective strategy to prevent FVIII-specific immune responses following gene therapy or protein replacement therapy. Our data also indicates that anti-CD3 can reduce the titers of pre-existing inhibitors and will be an excellent candidate in a combination therapy for modulating anti-FVIII immune responses in hemophilia mice with established inhibitors.
ACKNOWLEDGEMENTS:

We acknowledge the grant support from NIH/NHLBI (R01-HL069049 and R01-HL82600) and Bayer Hemophilia Foundation.

Authors’ contributions

B.P designed and performed research experiments, analyzed data, and wrote the paper. P.Y. performed experiments. D.J.R. revised the paper. H.D.O. provided helpful ideas and revised the paper. C.H.M. designed research project, analyzed data, and wrote the paper. Authors declare no competing financial interests.
REFERENCES


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Table 1. Absolute numbers of distinct populations of CD4+ T cells from FVIII plasmid+anti-CD3 and FVIII plasmid only treated mice at day 8, week 4 or week 6.

<table>
<thead>
<tr>
<th></th>
<th>Day 8</th>
<th>Week 4</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FVIII plasmid only</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ cells</td>
<td>(10.8±2.5) x 10^6</td>
<td>(11.0±1.8) x 10^6</td>
<td>(11.4±1.0) x 10^6</td>
</tr>
<tr>
<td>CD4*Foxp3+ cells</td>
<td>(1.43±0.20) x 10^6</td>
<td>(1.32±0.30) x 10^6</td>
<td>(1.35±0.30) x 10^6</td>
</tr>
<tr>
<td>CD4<em>CD25</em>Foxp3+ cells</td>
<td>(1.1±0.09) x 10^6</td>
<td>(1.0±0.24) x 10^6</td>
<td>(1.2±0.1) x 10^6</td>
</tr>
<tr>
<td>CD4<em>CD25</em>Foxp3+ cells</td>
<td>(1.7±0.37) x 10^5</td>
<td>(1.9±0.03) x 10^5</td>
<td>(1.8±0.23) x 10^5</td>
</tr>
<tr>
<td><strong>FVIII plasmid+anti-CD3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ cells</td>
<td>(4.2±1.1) x 10^6 *</td>
<td>(7.7±0.8) x 10^6 *</td>
<td>(10.4±1.5) x 10^6</td>
</tr>
<tr>
<td>CD4*Foxp3+ cells</td>
<td>(1.28±0.16) x 10^6</td>
<td>(1.38±0.13) x 10^6</td>
<td>(1.4±0.1) x 10^6</td>
</tr>
<tr>
<td>CD4<em>CD25</em>Foxp3+ cells</td>
<td>(0.74±0.17) x 10^6 *</td>
<td>(1.0±0.1) x 10^6</td>
<td>(1.1±0.08) x 10^6</td>
</tr>
<tr>
<td>CD4<em>CD25</em>Foxp3+ cells</td>
<td>(5.6±1.0) x 10^3 #</td>
<td>(2.1±0.02) x 10^3</td>
<td>(1.9±0.13) x 10^3</td>
</tr>
</tbody>
</table>

*, p<0.05; #, p<0.01 compared with data of FVIII plasmid only.
Table 2. Anti-CD3 treatment increases systemic TGF-β1.

<table>
<thead>
<tr>
<th></th>
<th>Systemic TGF-β1 (plasma: ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Naive mice</strong></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>15.2±0.99</td>
</tr>
<tr>
<td>#2</td>
<td>10.7±1.3</td>
</tr>
<tr>
<td>#3</td>
<td>8.6±1.4</td>
</tr>
<tr>
<td><strong>FVIII plasmid+anti-CD3 mice</strong></td>
<td></td>
</tr>
<tr>
<td>#4</td>
<td>Day3 43.25±2.28</td>
</tr>
<tr>
<td></td>
<td>Day10 39.97±0.27</td>
</tr>
<tr>
<td>#5</td>
<td>Day3 19.35±0.32</td>
</tr>
<tr>
<td></td>
<td>Day10 30.97±1.45</td>
</tr>
<tr>
<td>#6</td>
<td>Day3 7.56±0.35</td>
</tr>
<tr>
<td></td>
<td>Day10 15.44±0.66</td>
</tr>
</tbody>
</table>

Systemic TGF-β1 in the plasma was quantified by ELISA with duplicates. Day3 and day10 indicate days after the last anti-CD3 injection. Data of mean ± s.d. of individual mice are shown.
Table 3. Adoptive transfer of CD4+ cells from tolerized mice at week 6 rendered partial dominant tolerance to syngeneic mice.

<table>
<thead>
<tr>
<th>CD4+ cell donors</th>
<th>hFVIII activity of receivers (Naïve)</th>
<th>Anti-hFVIII antibodies of receivers (Bethesda Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day7</td>
<td>Day14</td>
</tr>
<tr>
<td>Naïve</td>
<td>317.0±76.2</td>
<td>2.2±2.3</td>
</tr>
<tr>
<td>FVIII Plasmid only</td>
<td>28.1</td>
<td>0</td>
</tr>
<tr>
<td>FVIII Plasmid+anti-CD3 (week 2)</td>
<td>331.1</td>
<td>12.5</td>
</tr>
<tr>
<td>FVIII Plasmid+anti-CD3 (week 6)</td>
<td>262.5±9.3</td>
<td>95.2±46.4</td>
</tr>
</tbody>
</table>

Cells were isolated from donors 2 weeks or 6 weeks after plasmid injection.
Table 4. Mice (n=4) were injected with 50 µg *FVIII* plasmid to induce anti-FVIII inhibitors. Eight week after plasmid injection, mice were treated with 40 µg anti-CD3 for 5 consecutive days. FVIII activities and anti-FVIII inhibitors were monitor prior and after anti-CD3 treatment as described in Material and Methods.

<table>
<thead>
<tr>
<th></th>
<th>Week 1 after <em>FVIII</em> plasmid injection</th>
<th>Week 4 after <em>FVIII</em> plasmid injection</th>
<th>Week 8 after <em>FVIII</em> plasmid injection</th>
<th>5 days of anti-CD3 treatment</th>
<th>Week 2 after anti-CD3 treatment</th>
<th>Week 4 after anti-CD3 treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FVIII activities ( % of normal)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>189</td>
<td>0</td>
<td>0</td>
<td>13.3</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>258</td>
<td>0</td>
<td>0</td>
<td>155.4</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>227</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>304</td>
<td>0</td>
<td>0</td>
<td>*</td>
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<table>
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<tr>
<th></th>
<th>Week 1 after <em>FVIII</em> plasmid injection</th>
<th>Week 4 after <em>FVIII</em> plasmid injection</th>
<th>Week 8 after <em>FVIII</em> plasmid injection</th>
<th>5 days of anti-CD3 treatment</th>
<th>Week 2 after anti-CD3 treatment</th>
<th>Week 4 after anti-CD3 treatment</th>
</tr>
</thead>
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<tr>
<td><strong>Anti-FVIII inhibitors (BU)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>0</td>
<td>0.3</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>0</td>
<td>50</td>
<td>62</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>0</td>
<td>0.3</td>
<td>1</td>
<td>0</td>
<td>0.1</td>
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</tr>
<tr>
<td>P4</td>
<td>0</td>
<td>45</td>
<td>88</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

* Mouse deceased.
FIGURE LEGENDS:

Figure 1. Long term FVIII expression in hemophilia A mice after FVIII plasmid-mediated gene therapy and immunomodulation with anti-CD3. Hemophilia A mice were treated with 50 µg of FVIII plasmid by hydrodynamic injection (n=4), or with 50 µg of FVIII plasmid at day 0 and 40 µg anti-CD3 by daily intravenous injection at days 0-4 (n=13). FVIII activities were assessed by a modified APTT assay and the anti-FVIII antibody titers by Bethesda assay over time. For plasmid only treated mice, (a) FVIII activity, (b) anti-FVIII antibody titers. For FVIII plasmid+anti-CD3 treated mice, (c) FVIII activity, (d) anti-FVIII antibody titers. Each symbol represents data obtained from an individual mouse.

Figure 2. Absence of recall proliferation in CD4+ T cells from FVIII plasmid plus anti-CD3 treated mice. CD4+ T cells were isolated by MACS from spleens of naïve, anti-CD3 only, FVIII plasmid only and FVIII plasmid+anti-CD3 treated mice 3 weeks after plasmid injection. 1.0 x 10^5 CD4+ T cells were co-cultured with irradiated 1.0 x 10^5 CD4− cells in 96-well round-bottom plates with or without the presence of FVIII at 10 U/ml for 72h, followed by adding 1 µCi [3H] thymidine per well for the final 18 hours. *, P<0.05, compared to the group of FVIII plasmid only. Data shown are mean ± S.D. of CPM of [3H] thymidine incorporation in triplicate wells.

Figure 3. Maintenance of immune tolerance to FVIII after a second challenge with FVIII plasmid. FVIII plasmid+anti-CD3 treated mice were given a second plasmid challenge at 23 weeks after the first plasmid injection. Three mice were chosen: two of these had persistent FVIII activity and one of these had lost FVIII activity. (a) FVIII activity, (b) anti-FVIII antibody titers were examined as described in Figure 1. Each line
represents an individual mouse. Data shown are from one representative experiment (2 independent experiments, n=3/group).

**Figure 4. Effect of anti-CD3 treatment on CD4\(^+\), CD8\(^+\) T cells, and CD4\(^+\)Foxp3\(^+\) Tregs in spleen and Blood.** (a-c) Spleen cells were isolated from \(FVIII\) plasmid only and \(FVIII\) plasmid+anti-CD3 treated mice (n=3/group). (a) Cells were stained and analyzed for CD4\(^+\), CD8\(^+\), and CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells by flow cytometry 8 days after the first anti-CD3 injection and \(FVIII\) plasmid transfer. Left panel shows the representative dot plots. Right panel shows summary of the data from two groups of mice. (b) Representative histograms of CD3 expression on CD4\(^+\)Foxp3\(^-\) and CD4\(^+\)Foxp3\(^+\) T cells obtained from \(FVIII\) plasmid only (left panel) and \(FVIII\) plasmid+anti-CD3 (right panel) treated mice. Dark line denotes CD4\(^+\)Foxp3\(^-\) cells, and light line denotes CD4\(^+\)Foxp3\(^+\) cells. (c) Detailed analysis of CD25 expression on CD4\(^+\)Foxp3\(^+\) Tregs at day 8, week 4, and week 6 after treatment. Upper panel, representative dot plots of Foxp3\(^+\) expression on CD4\(^+\) T cells (CD4\(^+\)Foxp3\(^+\) cells are gated), lower panel, representative dot plots of CD25\(^+\) expression on CD4\(^+\)Foxp3\(^+\) T cells (CD4\(^+\)CD25\(^{hi}\)Foxp3\(^+\), CD4\(^+\)CD25\(^{low}\)Foxp3\(^+\), and CD4\(^+\)CD25\(^{neg}\)Foxp3\(^+\) are each gated). Numbers are percentages of corresponding populations. Data shown are mean ± S.D.. **, p<0.01. (d-f) Blood samples were collected at serial time points from the four groups including naïve untreated, anti-CD3 only, \(FVIII\) plasmid only, and \(FVIII\) plasmid+anti-CD3 treated mice, stained and analyzed by flow cytometry for CD4\(^+\) T cells (d), CD4\(^+\)Foxp3\(^+\) Tregs (e), and CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs (f). Data shown are mean ± S.D.. **, p<0.01, compared to \(FVIII\) plasmid only; #, p<0.05, compared to anti-CD3 only.

**Figure 5. FVIII-specific suppressive capability of CD4\(^+\)CD25\(^+\) T cells derived from**
**FVIII plasmid+anti-CD3 treated mice.** For the FVIII specific suppressive assay, we used as responder CD4+ T (Tresp) cells from the spleen of a FVIII plasmid only treated mouse at week 4 after plasmid injection. CD4+CD25+ T cells from pooled splenic cells of naïve hemophilia A mice or FVIII plasmid+anti-CD3-treated mice were used as suppressive cells. The final co-culture system consisted of 0.8 x 10^5 CD4+Tresp cells, 1.5 x 10^5 irradiated CD4 cells, and CD4+CD25+ Treg cells at the indicated Treg:Tresp ratio. (a) CD4+CD25+ Tregs were isolated at week 2 after plasmid injection from mice treated with FVIII plasmid+anti-CD3. (b) CD4+CD25+ Tregs were isolated at week 6 after plasmid injection from mice treated with FVIII plasmid+anti-CD3. Data shown are mean ± S.D. of CPM of [3H] thymidine incorporation in triplicate wells.

**Figure 6. Effect of prior depletion of CD4+CD25+ T cells by anti-CD25 antibody upon tolerance induction.** (a) Diagram of anti-CD25 dosing schedule. (b) & (c) Anti-CD25 treatment depleted CD4+CD25+ T cells but not CD4+CD25FoxP3+ T cells in peripheral blood at day 8 after plasmid injection. (b) Representative dot plots of staining. (c) Summary of the data. (d) FVIII activities in hemophilia A mice treated with anti-CD25+FVIII plasmid+anti-CD3 (n=4/group) or with IgG1+FVIII plasmid+anti-CD3 control (n=4/group). One of two independent experiments is shown.

**Figure 7. Challenge of tolerized mice with unrelated T-dependent and T-independent antigens.** Tolerized mice (n=2) or naive mice (n=2) were challenged with the T-dependent antigen, bacteriophage Φx174 twice 4 weeks apart starting at week 24 after plasmid injection; antibody titers, expressed as Kv, were monitored as described in Materials and Methods. (a) Kv value over time following primary and secondary bacteriophage Φx174 immunization. The proportion of phage-neutralizing antibody of
the IgG isotype was measured 2 weeks post secondary immunization by treating with 2-ME and is provided in panel a. We used TNP-ficoll (20 µg) as T-independent antigen to immunize tolerized (n=4) or FVIII plasmid only (n=3) treated hemophilia A mice at week 24 after plasmid injection. Serum was collected at day 10 after challenge and assessed for (b) anti-TNP IgM, and (c) anti-TNP IgG3. No significant differences were found between the groups for Kv, anti-TNP IgM and anti-TNP IgG3 levels. Data are mean ± S.D.
Fig 1

a) FVIII activities (% of normal) over days after FVIII plasmid injection.

b) Anti-FVIII antibodies (Bethesda Units) over days after FVIII plasmid injection.

c) FVIII activities (% of normal) over days after FVIII plasmid injection.

d) Anti-FVIII antibodies (Bethesda Units) over days after FVIII plasmid injection.
Fig 4

(a) Flow cytometry histograms showing the percentage of CD4 or CD8+ T cells expressing FoxP3 or CD25 in different treatment groups. The histograms are labeled with the respective groups (Naive, FVIII plasmid, FVIII plasmid + anti-CD3).

(b) Bar graphs showing the percentage of CD4+ FoxP3+ cells at different time points: Day 8, Week 4, and Week 6 after plasmid injection. The graphs are labeled with the respective groups (FVIII plasmid, FVIII plasmid + anti-CD3).

(c) Table summarizing the percentage of CD4+ FoxP3+ cells at different time points:

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Group</th>
<th>CD4+ FoxP3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8 after plasmid injection</td>
<td>Naive</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>FVIII plasmid</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>FVIII plasmid + anti-CD3</td>
<td>27.7</td>
</tr>
<tr>
<td>Week 4 after plasmid injection</td>
<td>FVIII plasmid</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>FVIII plasmid + anti-CD3</td>
<td>21.0</td>
</tr>
<tr>
<td>Week 6 after plasmid injection</td>
<td>FVIII plasmid</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>FVIII plasmid + anti-CD3</td>
<td>13.2</td>
</tr>
</tbody>
</table>
Fig 5

(a) Week 2
Suppression (%)

- Tregs of naive control
- Tregs of FVIII plasmid+anti-CD3

Ratio of Treg to Tresp

(b) Week 6
Suppression (%)

- Tregs of naive control
- Tregs of FVIII plasmid+anti-CD3

Ratio of Treg to Tresp
**Fig 6**

### a

1 mg anti-CD25 (PC61) or IgG1

FVIII plasmid

Day 0 1 2 3 4 5 6 7 8 … 12 13 14 15

0.5 mg anti-CD25 (PC61) or IgG1

Anti-CD3 (40 µg/day for 5 days)

### b

<table>
<thead>
<tr>
<th>Condition</th>
<th>CD4⁺CD25⁺</th>
<th>CD4⁺FoxP3⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII plasmid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>anti-CD3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>anti-CD25</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IgG1 control</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

### c

Naive  IgG1 control  Anti-CD25

### d

FVIII activities (% of normal)

Days after FVIII plasmid injection

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Fig 7

(a) Graph showing the response over time for different treatments:
- **Naive (95% IgG)**
- **FVIII plasmid+anti-CD3 (100% IgG)**

Weeks after primary immunization:
- First immunization
- Second immunization

(b-c) Bar charts showing the comparison of anti-TNP levels:
- **b** FVIII plasmid vs. FVIII plasmid+anti-CD3
- **c** FVIII plasmid vs. FVIII plasmid+anti-CD3
Anti-CD3 antibodies modulate anti-factor VIII immune responses in hemophilia A mice after factor VIII plasmid-mediated gene therapy

Baowei Peng, Peiqing Ye, David J. Rawlings, Hans D. Ochs and Carol H. Miao