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Induction of long-term tolerance to a specific antigen using anti-CD3 lipid nanoparticles following gene therapy

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Development of factor VIII (FVIII) inhibitors is a serious complication in the treatment of hemophilia A (HemA) patients. In clinical trials, anti-CD3 antibody therapy effectively modulates the immune response of allograft rejection or autoimmune diseases without eliciting major adverse effects. In this study, we delivered mRNA-encapsulated lipid nanoparticles (LNPs) encoding therapeutic anti-CD3 antibody (αCD3 LNPs) to overcome the anti-FVIII immune responses in HemA mice. It was found that αCD3 LNPs encoding the single-chain antibodies (Fc-scFv) can efficiently deplete $CD3^+$ and $CD4^+$ effector T cells, whereas αCD3 LNPs encoding double-chain antibodies cannot. Concomitantly, mice treated with αCD3 (Fc-scFv) LNPs showed an increase in the $CD4^+$ $CD25^+Foxp3^+$ regulatory T cell percentages, which modulated the anti-FVIII immune responses. All T cells returned to normal levels within 2 months. HemA mice treated with αCD3 LNPs prior to hydrodynamic injection of liver-specific FVIII plasmids achieved persistent FVIII gene expression without formation of FVIII inhibitors. Furthermore, transgene expression was increased and persistent following secondary plasmid challenge, indicating induction of long-term tolerance to FVIII. Moreover, the treated mice maintained their immune competence against other antigens. In conclusion, our study established a potential new strategy to induce long-term antigen-specific tolerance using an αCD3 LNP formulation.

INTRODUCTION

Deficiency of coagulation factor VIII (FVIII) function results in a bleeding disorder called hemophilia A (HemA). Treatment of HemA patients requires FVIII protein replacement therapy or FVIII gene therapy to maintain plasma FVIII levels to prevent spontaneous or unexpected bleeding. However, around 25% of patients with severe HemA develop anti-FVIII antibodies with routine, repeated infusions of FVIII protein. Anti-FVIII antibodies can inhibit FVIII activity and/or promote clearance of FVIII.[1] It is essential to prevent or overcome the anti-FVIII immune responses to ensure the efficacy of therapeutic treatment of FVIII protein or gene therapy.

Anti-CD3 antibodies can effectively treat several autoimmune diseases in animal models.[2–5] Furthermore, humanized Ortho Kung T3 marketed as muromonab and several newly developed anti-CD3 mAbs such as otelixizumab, teplizumab, and visilizumab showed therapeutic effects for treating several autoimmune diseases including type 1 diabetes[6–9] and allogeneic transplant rejections.[10–12] Anti-CD3 mAb binds to TCR (T cell receptor)/CD3 complex to induce the internalization or shedding of TCR from the surface of T cells, resulting in anergy, apoptosis, and antigenic modulation of activated T cells. The different T cell subsets including $CD4^+$ effector T cells (Teffs) required for induction of an immune response and $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) for immunomodulation are affected disparately by anti-CD3 mAb treatment. Compared with Teffs, Tregs express significantly lower levels of the TCR/CD3 antigen receptor, CD3ϵ chains, TCR-ζ chain, or the CD4 coreceptor.[13] In addition, specific TCR/CD3 complexes in Tregs are linked to a higher activation threshold. Upon anti-CD3 mAb treatment, especially in the case of non-saturating anti-CD3 dosages, the Treg/Teff ratio increases to develop an environment that facilitates the induction of immune tolerance.[14]

Messenger RNA (mRNA)-based therapies have several advantages over recombinant protein replacement therapies and DNA-based gene transfer therapies, such that it can avoid aberrant post-translational modification or insertional mutagenesis of the host genome. An early application of mAb mRNAs was carried out by CureVac (EP 2101823 B1) in 2008,[15] along with other mRNA-based mAbs treatment regimens for passive vaccination, immunotherapy, and anti-toxin therapy.[16–18] Recent development of synthetic mRNA encapsulated in lipid nanoparticles (LNPs) provides a non-viral gene delivery method and has enabled significantly more efficient delivery of mRNA into specific cells and tissues.[19,20]

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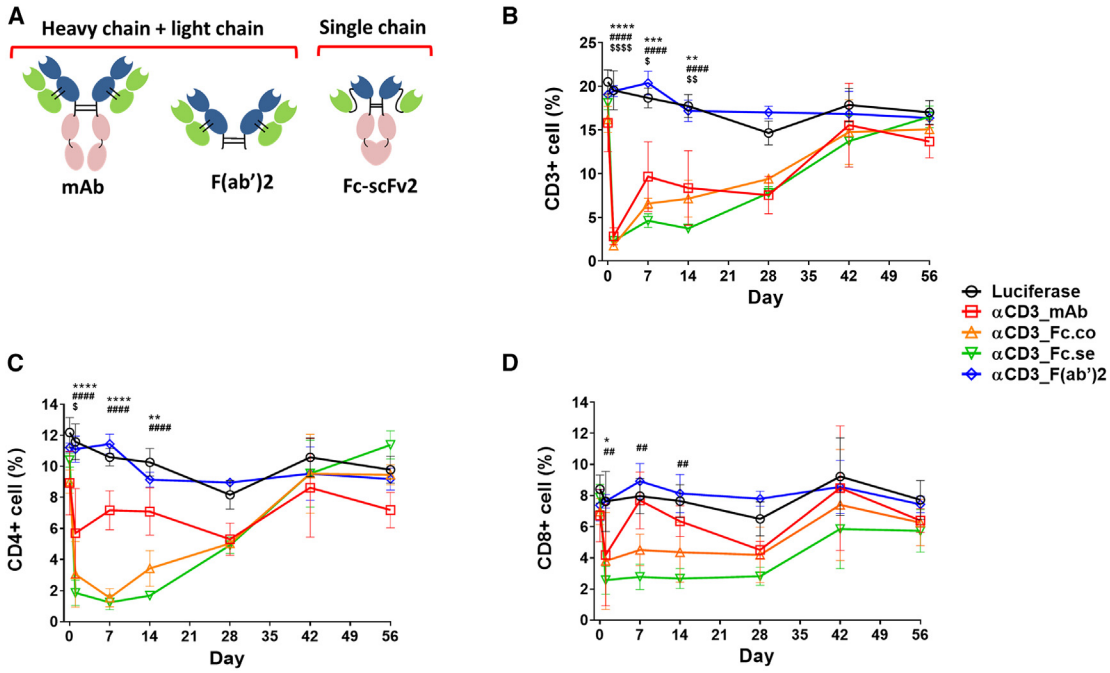

Figure 1. Different antibody formats of αCD3 LNPs induced differential depletion of T cell compartments

(A) mRNAs that encode different anti-CD3 antibody formats are encapsulated into lipid nanoparticles (LNPs). The αCD3_mAb(FL) is a full-length antibody composed of heavy chains (HCs) and light chains (LCs), whereas αCD3_F(ab')$_2$ does not contain an Fc region. αCD3_Fc-scFv.co (αCD3_Fc.co) and Fc-scFv.SE (αCD3_Fc.se) are composed of two single-chain variable fragments with an Fc region (Fc-scFv) and are designed according to different codon-optimized strategies. C57BL/6 mice (n = 3–4/group) were treated with 0.5 mg/kg αCD3 LNPs with different antibody formats, respectively. Percentages of different T cell compartments in PBMCs including (B) CD3$^+$ cells, (C) CD4$^+$ cells, and (D) CD8$^+$ cells were investigated by flow cytometry at different time points. Data are presented as average experimental values ± standard deviation. *Control vs. αCD3_Fc.co; #Control vs. αCD3_Fc.se; $Control vs. αCD3_mAb (*,$p < 0.05; **,##,$$p < 0.05; ***p < 0.001; ****,####,$$$$p < 0.0001).

In this study, we used an anti-CD3 mAb-encoding mRNA encapsulated in LNPs (αCD3 LNPs) to treat HemA mice prior to hydrodynamic injection of a liver-specific FVIII-expressing plasmid. Treatment with αCD3 LNPs significantly reduced the numbers of both CD4$^+$ and CD8$^+$ T cells while comparatively sparing Tregs, resulting in a substantial increase in the Treg/Teff ratio. Concurrent with the αCD3 LNP-induced effects on the Treg/Teff ratio, induction of anti-FVIII antibodies was prevented, resulting in persistent FVIII expression after FVIII plasmid-mediated gene therapy in HemA mice. In conclusion, αCD3 LNP treatment results in immune tolerance to FVIII protein in HemA mice, indicating the translational potential of mAb mRNA LNPs for immune modulation.

RESULTS

αCD3 LNPs can efficiently deplete T cells

To examine the immunomodulatory function of αCD3 LNPs, we first investigated if any change occurred in mouse T cell populations following αCD3 LNP treatment. Liver-targeted LNPs were employed for mRNA encapsulation. Delivery of control luciferase mRNA LNPs into the mice revealed that the bulk of mRNA expression was localized within the liver (Figure S1).[21] We first tested four different types of αCD3 mAb including the full-length (FL) mAb, two codon-optimized single-chain variable fragments (scFvs) with fragment crystallizable (Fc) region (Fc.co and Fc.SE), and an antigen-binding fragment (F(ab')$_2$) (Figure 1A). The mRNAs encoding heavy chains and light chains of FL or F(ab')$_2$ αCD3 mAb, were separately encapsulated into different LNPs, respectively, whereas the mRNAs coding for either Fc-scFv fragments were formulated into single LNPs. After injection of each αCD3 LNP at 0.5 mg/kg, peripheral blood mononuclear cells (PBMCs) were collected and CD3$^+$, CD4$^+$, and CD8$^+$ T cells compartments were examined by flow cytometry. αCD3_F(ab')$_2$ LNPs that encode anti-CD3 antibody without an Fc region did not exert any impact on CD3$^+$, CD4$^+$, and CD8$^+$ T cell numbers. However, mice treated with αCD3_mAb(FL), αCD3_Fc.co, and αCD3_Fc.se LNPs showed significant reductions in CD3$^+$ T cell numbers down to only 2.8%, 1.8%, and 2.3%, respectively, compared with 20% of CD3$^+$ T cells in control mice (Figure 1B). Both CD4$^+$ and CD8$^+$ T cells were depleted to significantly lower levels by αCD3_Fc.co and αCD3_Fc.se LNPs than αCD3_mAb(FL) LNP (1%–3% vs. 6%–7% and 3%–4% vs. 6%–7%, respectively) during the first 2 weeks following treatment (Figures 1C and 1D). αCD3_mAb(FL) LNP demonstrates comparable efficacy of depletion of CD3$^+$ T cells at a 2 mg/kg dosage; however, a delayed recovery period was observed (Figure S2). There is no difference between αCD3_Fc.co- and αCD3_Fc.se LNP-treated groups. After 6–8 weeks, T cells recovered to numbers comparable with the control mice. Since

our primary target cells were $CD4^+$ helper T cells that promote the development of anti-FVIII antibodies, we selected αCD3_Fc.co LNP (denoted as αCD3 LNP onward) in subsequent experiments as that mRNA formulation was the most effective in depleting $CD4^+$ T cells.

Depletion of T cells using αCD3 LNPs elevates percentage of Treg cells

Tolerance induction involves a balance shift, increasing the ratio of suppressive Tregs to Teffs. To examine whether αCD3 LNP treatment can induce antigen tolerance, we used HemA mice, which are prone to develop intense immune responses to human FVIII, as a tolerance induction animal model. HemA mice were injected with 0.5 mg/kg αCD3 LNPs twice on day −9 and −2 prior to challenge with human FVIII plasmid according to the therapeutic strategy (Figure 2A). Each mouse received 100 μg liver-specific hFVIII expression plasmid by hydrodynamic injection on day 0, and hFVIII activity and antigen expression level were examined at the indicated time points. First, the expression and secretion of plasma αCD3 mAbs were determined through western blot analysis (Figure 2B). The molecular weights of the detected proteins stand at 110 kDa dimer and 55 kDa monomer forms under non-reducing and reducing conditions, respectively. This observation indicates that the anti-CD3 Fc-scFv antibody can assemble into a functional dimeric structure via disulfide bonds. We next investigated the alteration of $CD3^+$, $CD4^+$, Teff, and Treg populations after αCD3 LNP treatment. As expected, mice treated with αCD3 LNPs showed decreasing cell populations of $CD3^+$ and $CD4^+$ cells from 32.7% and 22.5% to 2.0% and 1.1%, respectively, 1 day after the last treatment (Figures 2C and 2D). Furthermore, the percentage of $CD4^+CD25^+Foxp3^+$ Tregs dramatically increased from 3.8% to 15.8% and the Treg/Teff ratio exhibited about a 3-fold increase from pretreatment compared with the control mice, despite a 4.5-fold decrease in Treg cell numbers (Figure S3). These results suggest that the immunological setting had shifted to be more tolerogenic following αCD3 LNP treatment (Figures 2E and 2F). This Treg/Teff ratio gradually returned to the baseline ratio over a 1-month period.

We also examined a population of Treg precursor cells to further characterize the tolerogenic response. One week after αCD3 LNPs treatment, treated mice exhibited a dramatically higher cell population of alternative $CD4^+CD25^{low}Foxp3^+$ Treg precursor cells and a comparatively lower cell population of activated $CD4^+CD25^{high}$ $Foxp3^+$ Tregs when compared with the untreated mice, indicating an environment suitable for tolerance induction to specific antigens. We found that the percentages of precursor cells and activated Tregs reverted back to the normal levels over a 7-week period in αCD3 LNP-treated mice (Figure 3). It is expected that newly generated, activated $CD4^+CD25^{high}Foxp3^+$ Tregs would contain a high percentage of antigen-specific Tregs.[22,23]

Immune tolerance to hFVIII was established in HemA mice after αCD3 LNPs treatment

To examine whether treatment with αCD3 LNPs can result in immune tolerance to human FVIII in HemA mice, FVIII antigen level and activities of hFVIII plasmid-treated mice were examined by enzyme-linked immunosorbent assay (ELISA) and activated partial thromboplastin time (aPTT) clotting assay, respectively (Figures 4A and 4B). HemA mice challenged with FVIII plasmid only served as the control group. Following hFVIII plasmid-only treatment, mice generated high levels of FVIII expression (600%–800%) initially; however, the level rapidly dropped to less than 10% within 1 month. In contrast, following simultaneous treatment with αCD3 LNPs and hFVIII plasmid, mice generated a similarly high level of FVIII expression initially, with a slow decline to a therapeutic plateau level that was maintained for over 7 months. These results using αCD3 LNPs tolerance therapy were consistent with those obtained in immune-deficient HemA/NSG mice treated with FVIII plasmid (Figure 4C). In addition, αCD3 LNP-treated mice did not generate anti-FVIII antibody titers over the 7-month period compared with robust antibody development in control mice (Figure 4D). To further confirm the induction of immune tolerance to FVIII, the αCD3 LNP-treated mice received a second challenge of FVIII plasmid. All secondarily challenged mice had increases in both FVIII antigen and activity levels for 3 months post-challenge (Figures 5A and 5B). FVIII activity remained at the therapeutic plateau level out to endpoint day 535 (data not shown), demonstrating that long-term immune tolerance to FVIII was successfully induced by αCD3 LNP treatment.

Involvement of $CD4^+CD25^+$ Tregs in induction of immune tolerance to FVIII after αCD3 LNPs treatment

To examine whether $CD4^+CD25^+$ Tregs are involved in the induction of FVIII tolerance, splenocytes were harvested from tolerant mice and their Tregs were further isolated according to CD4 and CD25 markers. The isolated Tregs were co-cultured with irradiated $CD4^-$ cells from naive mice as antigen-presenting cells (APCs) and $CD4^+CD25^-$ cells from FVIII inhibitor mice as responder T cells (Tresps), and stimulated with recombinant FVIII for 4 days in an *in vitro* suppression assay. The Tregs from FVIII-tolerant mice more robustly suppressed proliferation of Tresps compared with naive Tregs at 1:1 and 1:2 Treg/Tresp ratios (Figure 6), indicating the presence of a greater number of FVIII-specific Tregs in αCD3 LNP-treated, tolerogenic mice.

HemA mice treated with αCD3 LNPs and hFVIII plasmid maintained immune competence against other antigens after αCD3 LNPs treatment

Since $CD3^+$ T cells were depleted in αCD3 LNP-treated mice, immune competence in treated mice was investigated after T cells had recovered to normal levels. A group of αCD3 LNP-treated mice were inoculated with trinitrophenylated keyhole limpet hemocyanin (TNP-KLH), unrelated T-dependent antigens mixed with complete Freund's adjuvant. Untreated naive HemA mice were inoculated with TNP-KLH as the control group. A secondary TNP-KLH challenge was administered 3 weeks after the first challenge. Peripheral blood plasma was collected and anti-TNP antibodies were measured by ELISA to evaluate the immune response. The αCD3 LNP-treated mice showed comparable antibody titers of

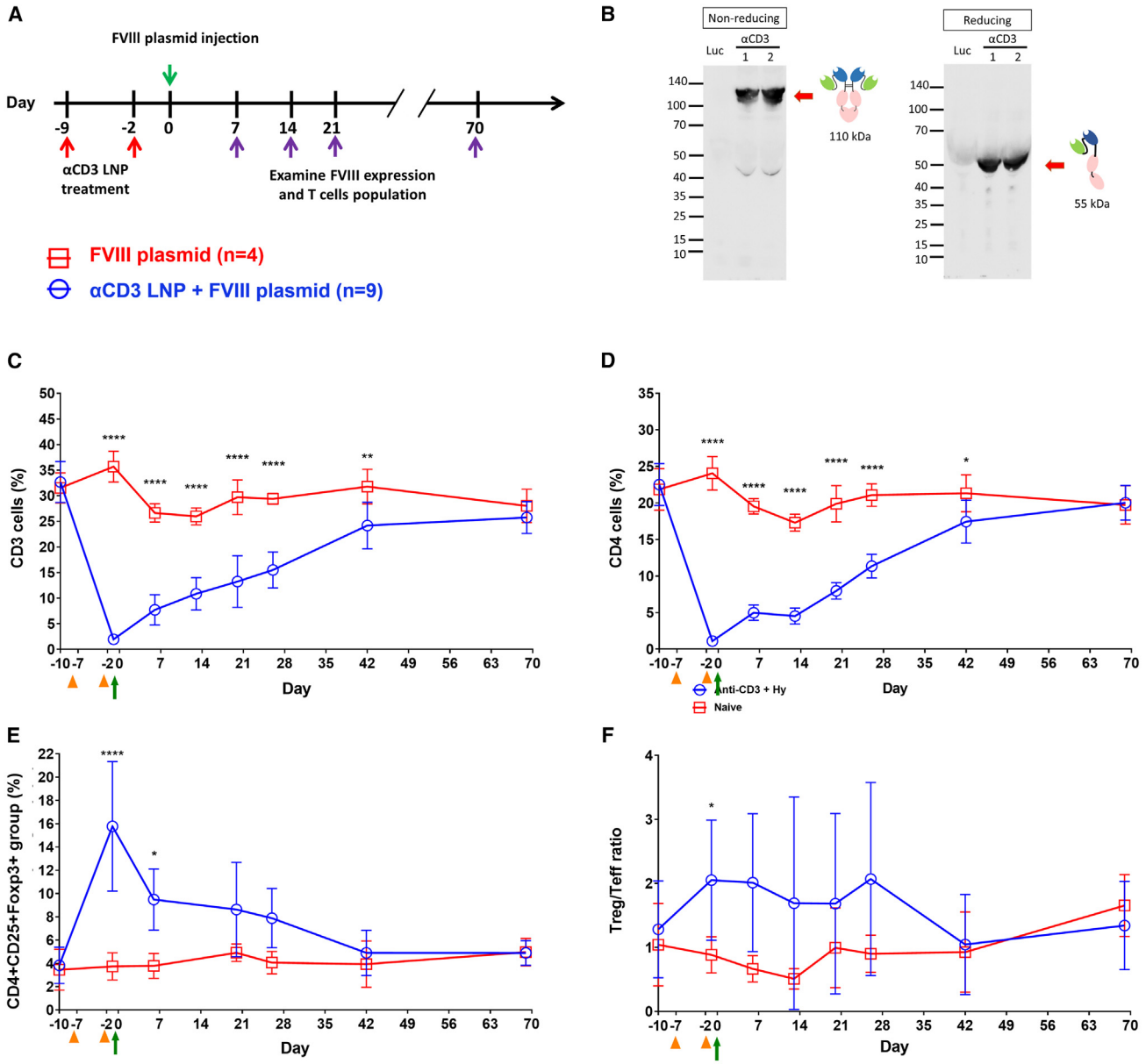

Figure 2. Injection of αCD3 LNPs LNP increases cell percentage of regulator T cells

(A) αCD3 LNP treating schedule of FVIII immune tolerance induction. Experimental mice were HemA mice treated with αCD3 LNPs and FVIII plasmid (n = 9). HemA mice treated with FVIII plasmid only served as control mice (n = 4). (B) Expression and secretion of anti-CD3 antibody after αCD3 LNP injection were confirmed by western blot. Plasma samples collected from luciferase LNP (Luc)-treated mice served as a control. Plasma samples from two αCD3 LNP-treated mice (lanes 1 and 2) were processed under non-reducing or reducing conditions and detected by anti-FLAG antibody, respectively. Arrows indicate the representation of anti-CD3 Fc-scFvs in dimeric or monomeric forms. Cell percentages of T cells in PBMCs at the indicated time points were analyzed by flow cytometry for (C) $CD3^+$ cells, (D) $CD4^+$ cells, (E) $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) of $CD4^+$ cells, and (F) Treg/$CD4^+CD25^+Foxp3^-$ effector T cell (Teff) ratio. Triangles indicate the injection days of αCD3 LNPs and arrows indicate hydrodynamic injection day of FVIII plasmid. Data are presented as average experimental values ± standard deviation (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

anti-TNP immunoglobulin M (IgM) and immunoglobulin G (IgG) levels compared with control HemA mice at all experimental time points (Figure 7). These results suggest that αCD3 LNP treatment did not permanently alter the immune competence against other antigens.

DISCUSSION

As of now, there are more than 100 mAbs in late-stage clinical studies or approved for clinical use by the US Food and Drug Administration (FDA) and/or the European Medicines Agency.[15] Production of mAbs is predominately performed in mammalian cell lines such as

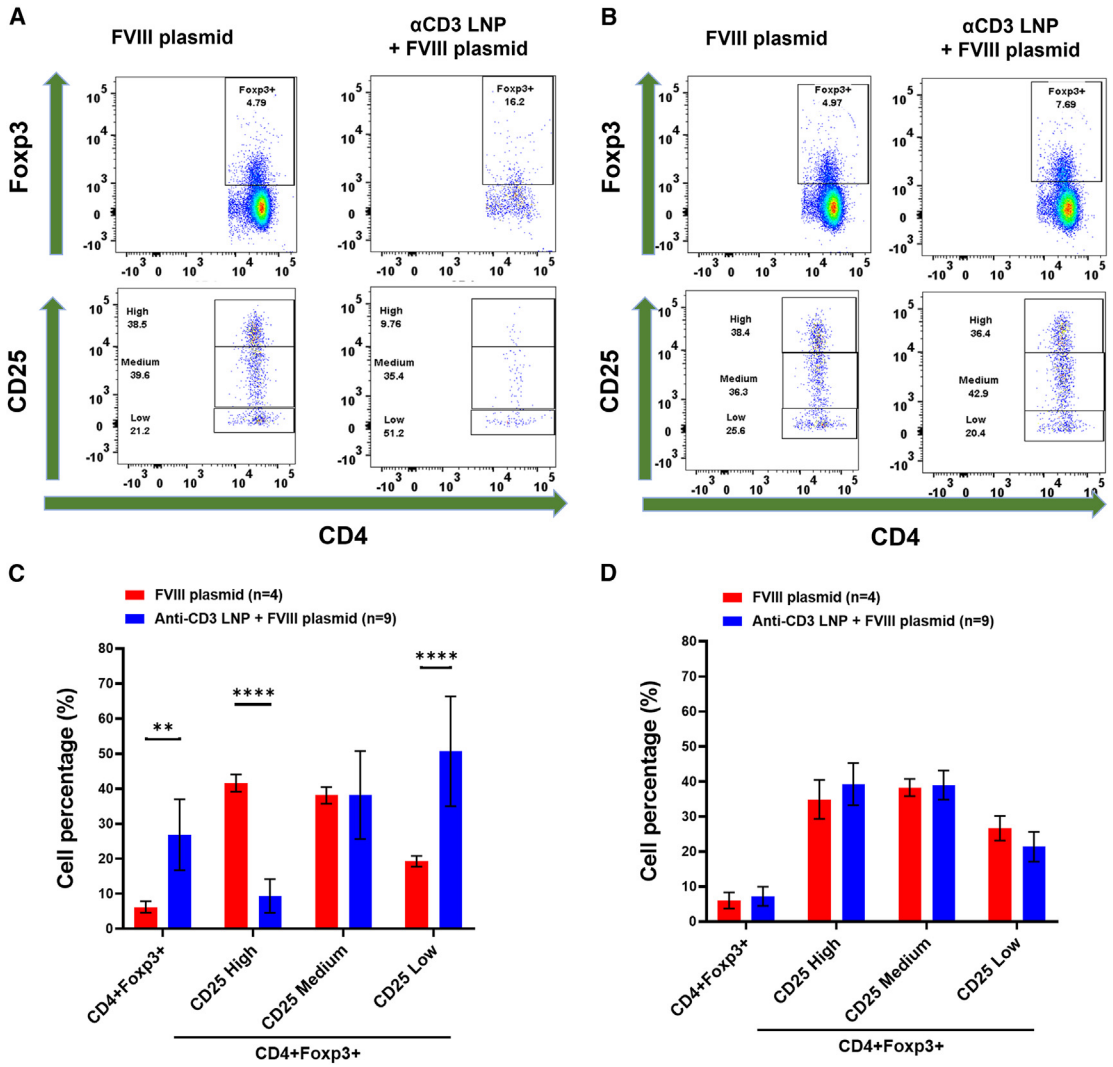

Figure 3. Immune modulation of activated Tregs

Cell population of Treg precursor cells was investigated in HemA mice treated with αCD3 LNPs and FVIII plasmid or HemA mice treated with FVIII plasmid only. Analysis of CD25 expression on $CD4^+Foxp3^+$ Tregs investigation was performed (A) 1 week and (B) 7 weeks after treatment. $CD4^+$ cells with $Foxp3^+$ expression were gated (top panel). The gated $CD4^+Foxp3^+$ cells were analyzed for different CD25 expression levels (bottom panel). Representative dot plots showed low, medium, and high expression of CD25 on $CD4^+Foxp3^+$ cells. Numbers are percentages of corresponding populations. Comparisons of different $CD4^+Foxp3^+$ populations between two groups (C) 1 week and (D) 7 weeks after treatment are shown (**$p < 0.01$, ****$p < 0.0001$).

embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO) and lower organism expression systems including insects, yeast, and bacteria. Expression of mAbs in these lower organisms has the advantage of high yield, lower cost, and the relative ease of manipulation compared with a mammalian expression system for manufacturing. However, most of these systems produce mAbs intracellularly and further purification steps from cell lysate or inclusion bodies are required. In addition, deficiency of post-translational glycosylation in bacteria and different glycosylation machinery in yeast and insect can result in higher immunogenic glycan structures compared with those found in humans.[24] Expression of mAbs in mammalian cell lines can provide more complete, biologically functional antibodies with proper protein folding and assembly of the heavy chain and light chain compared with lower organisms.[25] However, different glycosylation patterns in non-human cell lines may induce unexpected antibody responses in patients.[26–28] To date, several manufactured HEK293 cell lines were optimized for the production of mAbs with post-translational modification similar to natural human proteins for clinical use.[29–31] While effective for increasing antibody production, obstacles remain including the high cost of purification of mAbs due to potential aggregation of mAbs,[32] contamination of virus or serum proteins in cell culture medium,[33] and other toxicity concerns.

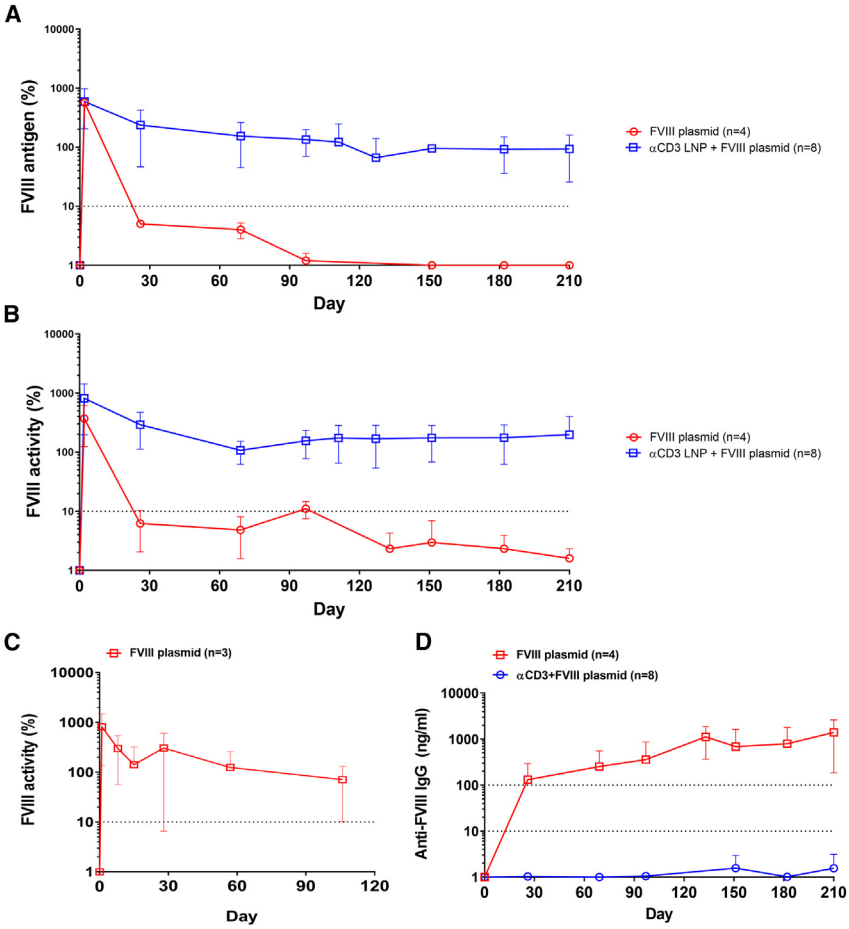

Figure 4. αCD3 LNP-treated HemA mice produced persistent expression of FVIII

HemA and NSG HemA mice received hydrodynamic injection of hFVIII plasmid after αCD3 LNP treatment. The treated HemA mice (n = 8) were examined for (A) FVIII antigen level and (B) FVIII activity using FVIII ELISA and aPTT assay, respectively. Mice not treated with αCD3 LNPs (n = 4) served as a control. (C) FVIII activity of NSG HemA mice (n = 3) was measured by aPTT assay after hydrodynamic injection. (D) The anti-FVIII antibodies were measured using anti-FVIII antibody ELISA. Data are presented as average experimental values ± standard deviation.

Recently, mRNA-based therapy has emerged in clinical therapeutics and has shown several superior features compared with traditional mAbs/proteins replacement or viral gene therapy. The therapeutic mAbs/proteins are synthesized in the cytoplasm of transfected cells and undergo natural post-translational modification without entering the nucleus. Thus, the mRNA modality ensures the synthesis of a natural protein form and prevents the risk of insertional mutagenesis associated with gene therapy approaches. Following delivery of the mRNA, functional mAbs/proteins can be rapidly made in a short time frame. By combining state-of-the-art techniques such as LNPs and nucleoside-modified mRNA, precise cell targeting and prevention of immune activation has been achieved in animal models.[34–36] Delivery of mRNA LNPs can be applied widely as a clinical treatment to replace current therapies including prophylactic vaccination and protein replacement therapies.[21,37–43] To date, therapeutic mAbs encoded by mRNA LNPs have been used and reported in numerous preclinical and clinical studies.[16,18,44–51] An mRNA LNP-based therapeutic mAb expression system has several potential advantages over recombinant mAbs for the treatment of patients. The variation of recombinant products produced in different cell systems can be diminished. Secondly, mAbs with natural post-translational modifications can maintain their functionality and reduce the risk of inducing potent immune responses against synthesized mAbs. In addition, mRNA removes the need of a purification step for mAbs from culture medium or cell extracts, which can lead to denaturation or low yield of recombinant mAbs.

In clinical practice using anti-CD3 mAb treatments, such as otelixizumab and teplizumab, consecutive administrations over 5–6 days are required to achieve optimal efficacy.[52,53] Conversely, antibody treatment facilitated by the mRNA LNP platform can manifest a more prolonged therapeutic impact due to endogenous mRNA expression compared with conventional administration of recombinant mAb proteins. In addition, *in vivo* expression of mRNA produces antibodies with appropriate post-translational modifications in host cells, reducing the potential toxicity caused by recombinant antibodies manufactured in CHO cells.[54] In recent developments, the approval of blinatumomab has garnered attention. This CD19/CD3 BiTE, designed within the innovative BiTE (bispecific T cell engager) format, has gained US FDA approval for addressing relapsed or refractory B cell precursor AML. Notably, blinatumomab's relatively short half-life of 2 h mandates continuous intravenous delivery for optimal outcomes. In contrast, the mRNA-LNP platform demonstrates the potential to achieve a compelling therapeutic response even at lower administration doses. This efficacy is attributed to the platform's capability to achieve heightened protein expression and prolonged antibody persistence, underscoring its advantages.[55]

Patients with severe HemA require routine prophylactic infusions of FVIII to prevent spontaneous bleeding. However, FVIII inhibitors produced from the immune response to infused FVIII recombinant protein or viral vector-expressed FVIII are major barriers to successful treatment for HemA patients. The current clinical treatment to modulate the anti-FVIII immune response is immune tolerance induction (ITI).[56–59] This approach requires HemA patients who develop FVIII inhibitors to receive repeated, daily to weekly infusions of high-dose FVIII protein for a long period of time. The duration of

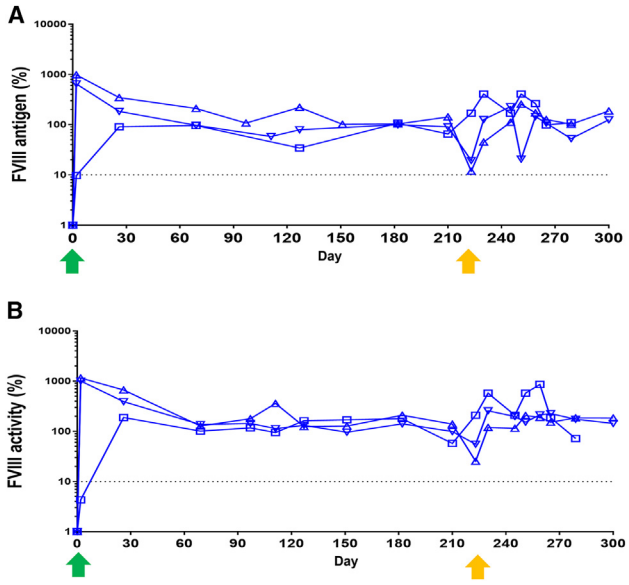

Figure 5. αCD3 LNP-treated HemA mice showed long-term immune tolerance to FVIII

HemA mice received a second hydrodynamic injection of FVIII plasmid 223 days after αCD3 LNP treatment. The treated mice were examined for (A) FVIII antigen level and (B) FVIII activity using FVIII ELISA and aPTT assay (n = 3), respectively. Arrows indicate the time of FVIII plasmid challenge (green, first injection; yellow, second injection).

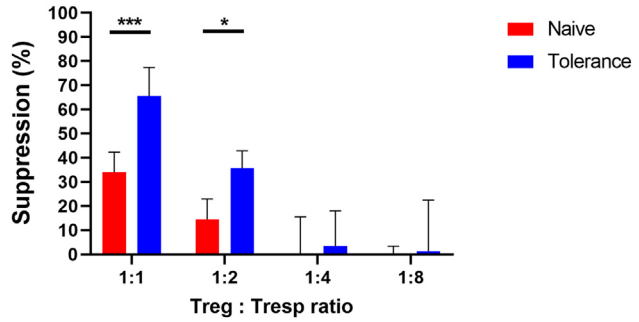

Figure 6. Isolated Tregs from αCD3 LNP-treated HemA mice showed FVIII-specific suppression

In vitro suppression assay was performed using HemA mice >4 weeks after αCD3 LNPs treatment and FVIII plasmid challenge. $CD4^+CD25^-$Teffs were isolated from splenocytes of FVIII inhibitor mice. $CD4^+CD25^+$ Tegs were isolated from splenocytes of αCD3 LNP-treated tolerant mice and naive mice, respectively. $CD4^-$ APCs of naive mice were isolated and irradiated. Teffs were co-cultured with the indicated ratio of Tregs from either tolerant mice or naive mice in the medium supplied with 2U FVIII and irradiated APC. The percentage of suppression was calculated from comparison of proliferation rate of $CD4^+$ cells between with or without addition of Tregs. The data are presented as means with standard deviation from three separate experiments (*$p < 0.05$, ***$p < 0.001$).

the therapy (ranges from 2 to 80 months) depends on different strategies and variance of patients to induce immune tolerance to FVIII. However, long-term tolerance to FVIII is still not achieved in 30%–40% of HemA patients after ITI. In addition, ITI is an invasive and costly therapy that decreases the quality of life for patients.

It has been recognized in studies for autoimmune disease and organ transplantation that approaches resulting in increases in the Treg/Teff ratio favored tolerance induction. This can be achieved by infusion of purified Tregs[60–63] or antigen-specific chimeric antigen receptor Tregs,[64,65] or *in vivo* expansion of Tregs by an IL-2/IL-2 analog.[66,67] Alternatively, decreasing Teff cell number by anti-CD3 antibody can also shift the balance of the Treg/Teff ratio.[68–70] In our previous investigations, we demonstrated that the administration of recombinant anti-CD3 mAb effectively modulates the immune response to FVIII, and these consistent outcomes have been observed across diverse study groups.[68,70] In this study, we investigated if administration of αCD3 mRNA LNPs into HemA mice can induce tolerance to FVIII. We first tested various mRNAs encoding different forms of anti-CD3 antibodies. Among four αCD3 LNPs we tested, our data demonstrated that αCD3_Fc.co and αCD3_Fc.se LNPs carrying single-chain anti-CD3 antibodies showed high efficiency in depleting both $CD4^+$ and $CD8^+$ T cells and eventual induction of FVIII-specific tolerance in treated animals. Despite previous studies showing that both recombinant FL anti-CD3_mAb and anti-CD3 $F(ab')_2$ fragments[71] could induce apoptosis of antigen-activated T cells, leading to antigen-specific tolerance, both αCD3_mAb(FL) and αCD3_$F(ab')_2$ LNPs were significantly less efficient in depleting $CD4^+$ and $CD8^+$ T cells. This discrepancy is probably due to lower efficiency and accuracy of the assembly of anti-CD3 antibodies since heavy chains and light chains of αCD3_mAb(FL) and αCD3_$F(ab')_2$ were separately formulated into LNPs. Similarly to our study, a lot of mRNA-based mAbs were designed by using Fc-scFv, heavy-chain-only V_H domains or nanobody form of antibodies that have smaller molecular weight and can be encapsulated in a single LNP compared with full-length mAb or $F(ab')_2$ to overcome this issue.[15,72] Another approach to improve this issue is to encapsulate the mRNAs of both the heavy chain and light chain into the same LNPs, ensuring their simultaneous targeting to the same cells and thereby increasing the accuracy of the assembly. An alternative approach to mitigating anti-FVIII antibodies involves oral delivery of anti-CD3 monoclonal antibody.[73] This method has the potential to modulate systemic immune responses, resulting in a reduction of anti-FVIII antibody titers, all without the need for T cell depletion. Further enhancements are anticipated, especially regarding the establishment of FVIII tolerance.

Following αCD3 LNP treatment, $CD4^+$ T cells including Tregs were significantly decreased, then slowly returned to normal levels during a 2-month period. Since Tregs express notably lower levels of the TCR/CD3 antigen receptor CD3ϵ chains, TCR-ζ chain, and the CD4 coreceptor than Teffs, anti-CD3 can more efficiently deplete Teffs than Tregs. A higher proportion of Tregs manage to persist following treatment with αCD3 LNPs, resulting in an elevated percentage of Tregs within the $CD4^+$ T cell population. During the recovery phase upon exposure to FVIII, the preferential increase of FVIII-specific Tregs may have favored the induction of FVIII-specific tolerance. Immediately after αCD3 LNP treatment, the percentage of $CD4^+CD25^{high}Foxp3^+$ Tregs strikingly decreased, resulting in

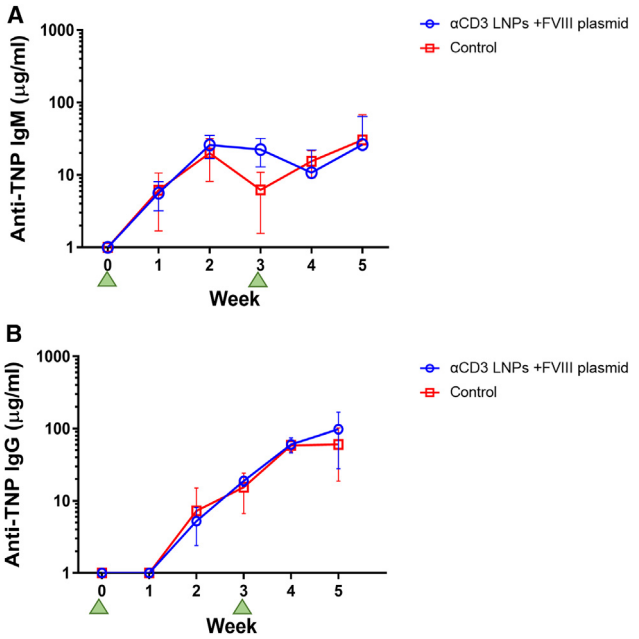

Figure 7. Immune competence of αCD3 LNP-treated HemA mice

Evaluation of immune competence was performed using trinitrophenyl (TNP) antigen challenge. Experimental mice (n = 5) were HemA mice 4 weeks after αCD3 LNPs treatment and FVIII plasmid challenge. Control mice (n = 5) were untreated naive HemA mice. All the mice were injected with 100 μg trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) mixed with an equal volume of complete Freund's adjuvant at weeks 0 and 3. Non-specific immune response was evaluated using (A) anti-TNP IgM (p = 0.713) (n = 5) and (B) anti-TNP IgG ELISA (p = 0.654) (n = 6). Triangles indicate the days of TNP-KLH challenge. The p values were calculated using two-way analysis of variance.

elevation of the percentage of $CD4^+CD25^{low/-}Foxp3^+$ Treg precursor cells. Subsequently, we observed gradual decreases in the percentage of $CD4^+CD25^{low/-}Foxp3^+$ Treg precursor cells and increases in the percentage of $CD4^+CD25^{medium/high}Foxp3^+$ Tregs. We suggest that induction of FVIII tolerance may result from the activation of new FVIII-specific Tregs from the $CD4^+CD25^{low/-}Foxp3^+$ Tregs population following αCD3 LNP and FVIII plasmid treatment. Second challenge with FVIII plasmid in αCD3 LNP-treated mice also suggested that long-term tolerance was induced, resulting in long-term FVIII expression. An *in vitro* suppression assay further demonstrated that the newly generated FVIII-specific Tregs suppressed the activation of $CD4^+$ Teffs in αCD3 LNP and FVIII plasmid-treated mice. This immune modulation induced by αCD3 LNP did not affect normal T cell-dependent immune responses since mice immunized with an unrelated antigen generated comparable antibody titers as in naive mice.

In conclusion, we successfully formulated αCD3 LNPs to produce therapeutic anti-CD3 antibodies. αCD3 LNP encoding an Fc-scFv fragment of the antibody is highly effective in suppressing anti-FVIII antibody production. Compared with multi-dose treatment using recombinant anti-CD3 antibodies, αCD3 LNP more efficiently depleted $CD3^+$ T cells with a dosing regimen 1–2 times higher. αCD3 LNP is a promising treatment regimen to induce long-term FVIII-specific tolerance in hemophilia patients.

MATERIALS AND METHODS

Synthesis of αCD3 LNP

αCD3 LNPs were formulated according to previous methods.[21,74] The αCD3 mRNA sequence with FLAG tag is synthesized based on anti-CD3 epsilon (clone 145-2C11). Different types of αCD3 LNP mRNA were synthesized *in vitro* using an optimized T7 RNA polymerase-mediated transcription reaction with complete replacement of uridine by N1-methyl-pseudouridine.[35] The reaction included a DNA template containing the immunogen open reading frame flanked by 5′ untranslated region (UTR) and 3′ UTR sequences and was terminated by an encoded poly(A) tail. After transcription, the Cap 1 structure was added to the 5′ end using vaccinia capping enzyme (New England Biolabs) and Vaccinia 2′ *O*-methyltransferase (New England Biolabs). mRNA LNPs composed of ionizable lipid, DSPC, cholesterol, and PEG-lipid (50:10:38.5:1.5) were assembled using the NanoAssemblr system (Precision NanoSystems, San Francisco, CA) with synthetic mRNAs at a volume ratio of 1:3. The drug product underwent analytical characterization, which included the determination of particle size and polydispersity, encapsulation, mRNA purity, double-stranded RNA content, osmolality, pH, endotoxin, and bioburden, and the material was deemed acceptable for *in vivo* study.

Animal

HemA mice that have targeted disruption of exon 16 in the FVIII gene with a mixed genetic background of 129/SV × C57BL/6 at the age of 8–12 weeks were used in these experiments. All the experimental mice were housed at a specific pathogen-free facility in Seattle Children's Research Institute according to the animal care guidelines of the National Institutes of Health and Seattle Children's Research Institute. The experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Seattle Children's Research Institute.

LNP injection and plasma sample collection

αCD3 LNPs were intravenously injected to HemA mice through the retro-orbital plexus according to experimental design. Blood samples collected from the retro-orbital plexus from treated HemA mice at the indicated time points were centrifuged immediately at 500 × *g* for 5 min to obtain plasma. Plasma samples were aliquoted and stored at −80°C for further experiments.

Flow cytometric analysis of PBMCs

PBMCs were isolated from whole blood collected in plain capillary tubes containing 3.8% buffered sodium citrate. After lysis of red blood cells by ammonium-chloride-potassium lysing buffer, PBMCs were first stained with surface markers of PerCP-Cy5.5 rat anti-mouse CD3 molecular complex (Fisher Scientific, WA, BDB560527), Alexa Fluor 700 anti-mouse CD4 (Fisher Scientific, BDB557956), and PE anti-mouse CD25 (102008, BioLegend San Diego, CA), and subsequently intracellular staining was carried out with Alexa Fluor 647

anti-mouse Foxp3 (Fisher Scientific, BDB560401) with Foxp3/transcription factor staining buffer set (Fisher Scientific, 50-112-8857). Flow cytometric analysis of stained cells was performed on the BD LSR II.

Western blot

Plasma samples were subjected to electrophoresis on a 4%–15% SDS-PAGE gel (Bio-Rad) and then transferred to polyvinylidene fluoride membranes using an iBlot2 gel transfer device (Thermo Fisher Scientific). These membranes were subsequently blocked with non-fat skimmed milk. FLAG-tagged Anti-CD3 Fc-scFv was detected using a rabbit anti-FLAG antibody (Thermo Fisher Scientific, A00170-40), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific). Enhanced chemiluminescence (ECL) signals were developed using ECL reagent (Thermo Fisher Scientific).

aPTT assay

Plasma was isolated from whole blood collected using plain capillary tubes containing 3.8% buffered sodium citrate. Plasma hFVIII was examined using a modified clotting assay utilizing aPTT reagent and FVIII-deficient plasma[75,76] on a Stago Compact instrument. hFVIII activity was evaluated according to a standard curve obtained from serially diluted normal human pooled plasma.

hFVIII and anti-hFVIII antibody ELISA

hFVIII antigen levels in plasma of experimental mice were examined by ELISA using murine anti-FVIII antibody (GMA-8020, Green Mountain Antibody, Burlington, VT) and biotin-labeled murine anti-FVIII antibody (Green Mountain Antibody, GMA-8015).[21] Serially diluted normal human plasma pool was used as standards to evaluate human FVIII antigen level. Anti-hFVIII IgG levels were examined by inhibitor ELISA. Recombinant human FVIII (Kogenate FS, Bayer, NJ) was used as antigen for detection of anti-hFVIII antibodies. Recombinant FVIII protein were used to coat 1 unit per well in 0.1 M sodium bicarbonate buffer (pH 9.7) overnight at 4°C. TBS (40 mM Tris, [pH 7.5], 150 mM NaCl) buffer with 5% non-fat milk was used as blocking buffer for 2 h at room temperature and the plates were washed with 0.005% Triton X-100 TBS washing buffer. Plasma samples from experimental mice were diluted with blocking buffer added to the wells and incubated overnight at 4°C. HRP-conjugated goat anti-mouse IgG antibodies (Thermo Fisher Scientific, 31436) was then used to detected anti-hFVIII IgG.

T cell suppression assay

FVIII inhibitor mice were generated by co-injection of 20 μg of lipopolysaccharide (Sigma-Aldrich) and 3U FVIII three times per week for 4 weeks. The FVIII inhibitor titer was determined by anti-hFVIII antibody ELISA. $CD4^+$ T cells and APCs ($CD4^-$ cells) were isolated from FVIII inhibitor mice, FVIII-tolerant mice and naive mice by mouse $CD4^+$ T cell isolation kit (Miltenyi Biotec, Germany, 48237) using autoMACS Pro Separator (Miltenyi Biotec). $CD4^+CD25^-$ cells (Teff) and $CD4^+CD25^+$ cells (Treg) subsets were further isolated by mouse $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec, 49431). For suppressive assay, 1×10^5 Teffs isolated from inhibitor mice were pre-treated with Fixable Viability Dye eFluor 450 (Fisher Scientific, 50-112-8817) and co-cultured with 2×10^5 irradiated APCs isolated from naive mice, and indicated ratios of cell number of Tregs isolated from tolerant mice or naive mice. FVIII (10 U/mL) was added to culture medium to stimulate proliferation. All cultures were performed in triplicate. Cells were stained with Fixable viability stain 575V (Fisher Scientific, BDB565694) and the indicated surface cell markers. Percentages of cell proliferation were analyzed using flow cytometry on the BD LSR II. Suppression efficiency was calculated as follows:

$$\%\ \text{suppression} = [1 - (\%\ \text{proliferation of Treg + Tresp})/(\%\ \text{proliferation of Tresp alone})] \times 100\%$$

Immunization by non-specific challenge with TNP antigen

αCD3 LNP-treated FVIII tolerant mice and naive mice were intraperitoneally immunized with 100 μg TNP-KLH (Fisher Scientific, NC9810752) or 25 μg TNP-Ficoll (Fisher Scientific, NC9092506,) with complete Freund's adjuvant. A second challenge of TNP antigen was injected 3 weeks later with the same dosages. Anti-TNP antibody ELISA was performed in plates coated with TNP-bovine serum albumin (Biosearch Technologies, CA, T-5050-10) as described above. Anti-TNP IgG, IgG3, and IgM titers were detected using HRP-conjugated anti-mouse IgG, IgG3 (Fisher Scientific, M32607,) and IgM (Thermo Fisher Scientific, 31440) antibodies.

Statistical analyses

All the statistical analyses were carried out utilizing GraphPad Prism 7 software. The data were compared using two-tailed unpaired Student's t test and one- or two-way analysis of variance followed by post hoc Bonferroni's multiple comparison tests. p values < 0.05 were considered statistically significant.

DATE AND CODE AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author Carol Miao, carol.miao@seattlechildrens.org.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.102043.

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AUTHOR CONTRIBUTIONS

C.-Y.C. developed study design, performed experiments, analyzed data, and wrote the manuscript. A.V.K. performed experiments and reviewed the manuscript. A.C. and X.C. performed experiments. J.H. reviewed and revised the manuscript. P.G.V.M. reviewed the manuscript. C.H.M. developed the study concept, analyzed results, and wrote and revised the manuscript.

DECLARATION OF INTERESTS

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