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Long-term tolerance to factor VIII is achieved by administration of IL-2/IL-2mAb complexes and low dosages of factor VIII

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Summary

Background—Regulatory T cells (Tregs) play a pivotal role in regulating anti-factor VIII (FVIII) immune responses. Interleukin (IL)-2 mixed with a particular IL-2 monoclonal antibody (mAb; JES6-1) can induce selective expansion of Tregs *in vivo*.

Methods—In the prevention experiments, we treated hemophilia A mice with IL-2/IL-2mAb complexes (3x/week), and concurrently with FVIII protein (80U/kg/week) for 4 weeks. Generation of anti-FVIII immune responses was examined afterwards. Next, in order to induce long-term tolerance to FVIII, a series of treatment dosages and schedules for administering IL-2/IL-2mAb complexes and FVIII protein in hemophilia A mice was evaluated.

Results—Compared to control FVIII only treated mice which produced high-titer anti-FVIII antibodies, mice treated with IL-2/IL-2mAb complexes + FVIII produced no antibodies. A marked 7 fold increase in CD4⁺CD25⁺Foxp3⁺Helios⁺ natural Tregs was maintained for 4 weeks in blood, spleen and lymph nodes, and dropped to normal levels within the next 10 days. The suppressive functions of expanded Tregs were demonstrated by suppressive, proliferative and cytokine assays. Administration of anti-CD25 mAb (PC-61) blocked this protective effect, confirming the involvement of Tregs in suppressing anti-FVIII immune responses. Importantly, administration of IL-2/IL-2mAb complexes (3x/week for 8 weeks) combined with contiguous weekly injections of low dosage FVIII protein (20U/kg/week for 18 weeks) not only abrogated the formation of anti-FVIII antibodies, but also induced long-term tolerance to FVIII.

Conclusions—Treatment of IL-2/IL-2mAb complexes is highly promising for induction and maintenance of FVIII-specific tolerance following FVIII protein replacement therapy.

Keywords

IL-2; anti-IL2 antibody; factor VIII; hemophilia; immune tolerance; immunomodulation; regulatory T cell; protein replacement therapy

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Explanation of Author Contributions:

C. L. Liu designed and performed research, analyzed data, and wrote the paper. P. Ye performed research. J. Lin performed research. D. Djukovic developed and performed experiments to measure kynurenine levels and wrote the analytical method. C. H. Miao designed the project and research, analyzed data, and wrote the paper.

Conflict of Interest Disclosure:

The authors have no conflict of interest to declare.

INTRODUCTION

Hemophilia A is an inherited, X-linked, recessive disorder caused by deficiencies of functional plasma clotting factor VIII (FVIII). In clinical practice, approximately 25–30% of all treated hemophilia A patients develop inhibitory anti-FVIII antibodies (Abs; FVIII inhibitors), which significantly increase morbidity and lower the quality of life. Anti-FVIII antibodies neutralize the procoagulant function of FVIII[1] and represent the greatest limitation to successful FVIII replacement therapy. Bypassing agents[2] and current immune tolerance induction protocols (ITI)[3–5] are very expensive or take a long time to complete and sometimes these strategies are unsuccessful for some patients. As a result, new strategies to induce immune tolerance to FVIII have attracted much research interest in recent years.

There has been some successful development of immunomodulation protocols to regulate FVIII inhibitors in animal models (see reviews[6, 7]). Interestingly, many of these protocols involve the induction of activated regulatory T cells (Tregs)[8]. Tregs comprise a crucial T-cell compartment to maintain peripheral tolerance by suppressing autoreactive T-cell responses[9] as well as orchestrating a balanced immune response to foreign antigens[10]. Tregs can suppress a variety of immune cells[11] including B cells, NK cells, CD4⁺ and CD8⁺ T cells, as well as monocytes and dendritic cells (DCs). Immunosuppressive cytokines including Interleukin (IL)-10, IL-35 and TGF- β produced by the activated Tregs are also involved in the suppressive function of Tregs. IL-2, a T cell growth factor, is essential for the growth and development of Tregs[12] since acute neutralization of IL-2 in adult mice disrupts Treg homeostasis[13]. Recently, IL-2 bound to a specific anti-IL-2 monoclonal antibody (mAb; JES6-1) (denoted as IL-2/IL-2mAb complexes) was shown to selectively and significantly expand CD4⁺CD25⁺ Tregs *in-vivo*[14]. Administration of IL-2/IL-2mAb complexes regulated immune responses in several different model systems[14, 15], such as inducing resistance to experimental autoimmune encephalomyelitis (EAE) and promoting long-term acceptance of pancreatic islet allografts.

In this study, we adopted the immunomodulation approach using IL-2/IL-2mAb complexes to regulate FVIII-specific immune responses following FVIII replacement therapy. Administration of IL-2/IL-2mAb complexes induced a five- to seven-fold expansion of Tregs transiently in the peripheral blood, LNs and spleens of the treated mice. A series of treatment protocols were evaluated. We found that both factor VIII dosages and IL-2/IL-2mAb complexes treatment duration are very important factors to induce and maintain long-term tolerance to factor VIII in hemophilia A mice.

METHODS

Mice

All mice were kept according to the animal care guidelines of National Institutes of Health and Seattle Children's Research Institute. Hemophilia A mice in a 129/SV $\times$ C57BL/6 mixed genetic background were generated by targeted disruption of exon 16 of *FVIII* gene[16] and were used at the age of 6–8 wks.

Treatment of hemophilia A mice with FVIII protein transfer and immunomodulation using IL-2/IL-2mAb complexes

Hemophilia A mice were injected with FVIII protein (Kogenate®, Wayne, NJ) at different dosages (see Table 1) in 200 µl phosphate-buffered saline via tail vein injection. IL-2/IL-2mAb complexes were prepared as previously described[15, 17]. One µg recombinant mouse IL-2 (Pepro Tech, Rocky Hill, NJ) was mixed with 5 µg anti-IL-2 mAb (JES6-1A12) (eBioscience, San Diego, CA), incubated at 37°C for 30 minutes, and injected intraperitoneally (i.p.) into mice according to schedules specified in Table 1. FVIII protein only treated and naive hemophilia A 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.

Depletion of Tregs by anti-CD25mAb

Hemophilia A mice were treated with IL-2/IL-2mAb complexes + FVIII + PC-61 (anti-CD25 mAbs; Bio Express, West Lebanon, NH). Mice (n=4) received 1 mg of PC-61 i.p. at 1 and 14 days each after the last IL-2/IL-2mAb complexes and FVIII injection[18]. Depletion of CD4⁺CD25⁺ cells was examined by flow cytometric analysis of collected blood samples.

Flow cytometry and antibodies

Cell suspensions were stained for fluorescence-activated cell sorting (FACS) analysis using the following antibodies [obtained from eBioscience (San Diego, CA) unless otherwise stated][1]: PE-Cy5- anti-mouse CD25; FITC- anti-mouse Helios (BioLegend, San Diego, CA); Alexa Fluor® 647- anti-mouse/rat Foxp3; PE- anti-mouse cytotoxic T lymphocyte antigen 4 (CTLA-4); Alexa Fluor® 700- anti-mouse CD4 and PE-Cy7- anti-mouse glucocorticoid-induced TNFR (GITR; BD Pharmingen™, San Jose, CA). Cells were stained for surface markers CD4, CD25, and GITR, and intracellularly for Foxp3, Helios and CTLA-4 following the company protocol (eBioscience). Samples were analyzed using an LSRII flow cytometer (Becton Dickinson, Palo Alto, CA) and FlowJo software (Tree Star, Ashland, OR).

FVIII activities and inhibitor titer assays

Peripheral blood samples were collected from the experimental mice within 15 minutes after FVIII infusion. The activated partial thromboplastin time (APTT) was measured by a modified clotting assay using FVIII deficient plasma[19]. Anti-FVIII activities were measured by Bethesda assay as previously described[19]. Serum anti-FVIII specific IgG1 concentrations were detected using enzyme-linked immunosorbent assay (ELISA).

Proliferative and suppressive assays

CD4⁺ T cells were isolated from spleens of mice by magnetic activated cells sorting (Miltenyi Biotec, Auburn, CA). The CD4⁺CD25⁻, CD4⁺CD25⁺ subsets were further purified from the CD4⁺ T cells using a CD25⁺ Treg MACS isolation kit (Miltenyi Biotec). For proliferation assay, 1.0×10^5 CD4⁺ cells were incubated in the presence of 1.0×10^5 CD4⁻ cells (irradiated, used as antigen presenting cells) per well and stimulated with FVIII at 10U/ml (1U = 100 ng FVIII protein) for 72 hours, followed by adding 1µCi

[³H]thymidine (PerkinElmer; Boston, MA) for the final 18 hours. [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 mice treated with FVIII protein only were used as responders (Tresp) and CD4⁺CD25⁺ T cells from tolerized or naïve mice at different time points were added as suppressor cells. To the co-culture of 0.8×10^5 CD4⁺ T cells and 1.5×10^5 antigen presenting cells, we added CD4⁺CD25⁺ T cells at indicated ratios. Suppression was calculated as [18]:

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

Quantitation of TGF-β levels by ELISA

TGF-β concentrations were quantified in plasma pretreated with 1N HCl, and then assayed using the ELISA kit (BD Biosciences, San Jose, CA) according to the manufacturer's recommendation.

Immunization with the T-dependent antigen TNP-KLH

Groups of tolerized and naïve mice (n=3/group) were immunized with 100 µg TNP-KLH (Biosearch Technologies, Inc.) emulsified in Freund's complete adjuvant (Sigma, Missouri, USA) i.p. 22 weeks after FVIII protein infusions. A second boost was administered 3 weeks later. Mice were bled every 7 days and serum samples were analyzed for anti-TNP IgM and IgG Abs by ELISA in the plate coated with trinitrophenyl-bovine serum albumin (TNP-BSA; Biosearch Technologies, Inc.).

Kynurenine levels analysis

Plasma kynurenine levels in treated and control naïve hemophilia mice were determined at the indicated time points using High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) by an adapted method from University of Washington Nutrition Obesity Research Center (NORC) Metabolomics Subcore. The LC system was composed of a Agilent 1260 binary pump, an Agilent 1260 auto-sampler, and Agilent 1290 column compartment (Agilent Technologies, Santa Clara, CA). Injection volume was 2 µL. Chromatographic separations were performed in hydrophilic interaction chromatography (HILIC) mode on SeQuant ZIC-cHILIC column (Merck KGaA, Darmstadt, Germany) with a flow rate at 0.3 mL/min. The identities of L-kynurenine and L-tyrosine were confirmed by spiking the pooled serum sample (QC sample) with a mixture of standard L-kynurenine and L-tyrosine. After the chromatographic separation, MS ionization and data acquisition were performed using an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) equipped with an electrospray ionization (ESI) source working in positive ionization mode.

Statistical analysis

Results are presented as means ± SD. The statistical significance of the difference between means was determined using the two-tailed Student's *t* test. Differences were considered significant at *P* < 0.05.

RESULTS

IL-2/IL-2mAb complexes treatment prevented formation of anti-FVIII antibodies following FVIII protein replacement therapy

Hemophilia A mice (n=7) were treated with IL-2/IL-2mAb complexes (3x/week), and concurrently with FVIII protein (80U/kg/week) for 4 weeks (Fig. 1a). Plasma samples were collected from the experimental and naive mice at scheduled time points to assess the inhibitory antibody titers. As shown in Fig. 1b, left panel, mice treated with IL-2/IL-2mAb complexes + FVIII produced no inhibitory antibodies. However, all control mice treated with FVIII only (n=3, Fig. 1b, right panel) generated high-titer inhibitory antibodies starting on week 4 and the titers persisted at high levels afterwards. Furthermore, high levels of anti-FVIII IgG1 were detected in the FVIII only treated group compared to undetectable levels of anti-FVIII IgG1 in IL-2/IL-2mAb complexes + FVIII treated and naive mice at 4–6 weeks after treatment (Fig. 1c).

IL-2/IL-2mAb complexes treatment selectively increased the percentages and activities of CD4⁺CD25⁺Foxp3⁺ Tregs in peripheral blood, Spleen and LNs

Next, we investigated if changes in T cell populations of hemophilia A mice correlate with immunomodulation by IL-2/IL-2mAb complexes treatment. We first analyzed peripheral blood in selected mice over treatment period. As shown in the dot plot figure, no significant difference of CD4⁺ T cell population was observed (Fig. 2a, left panel) between naive and IL-2/IL-2mAb complexes treated mice, however, the CD4⁺CD25⁺Foxp3⁺ T cells (Fig. 2b, left panel) as well as CD4⁺Foxp3⁺Helios⁺ T cells (Fig. 2c, left panel) were about 7 to 8-fold increased in the IL-2/IL-2mAb complexes treated mice compared to the naive mice on the peak day of Treg expansion. A detailed flow cytometry analysis showed that the percentage of CD4⁺ T cells did not change over time (Fig. 2a, right panel; $P < 0.05$) between all the mouse groups; however, the percentage of CD4⁺CD25⁺Foxp3⁺ T cells within the CD4⁺ T cell compartment remain at significantly increased levels in the treated mice compared to the control mice over the 4 week treatment period (Fig. 2b and 2c, right panel). Furthermore, the percentages of CD4⁺Foxp3⁺Helios⁺ T cells were also proportionally increased as the CD4⁺CD25⁺Foxp3⁺ T cells in the IL-2/IL-2mAb complexes treated mice (Fig. 2c, right panel), indicating most of the expanded cells originated from Helios⁺ natural Tregs. The expanded Foxp3⁺Helios⁺ Tregs then declined gradually to background levels by week 7. Additionally, considerable higher expression levels of molecules crucial to the suppressive function of Tregs were observed during Treg expansion period, including CD25, GITR, and CTLA-4 as indicated by their (Mean Fluoresces Index (MFI)) levels (Fig. 2d). Similar trend of Treg expansion and maintenance of CD4⁺ T cell populations following the injection of IL-2/IL-2mAb complexes was also observed in spleen (Supplemental Fig. 1) and LNs (Supplemental Fig. 2).

Examination of the roles of effector T (Teff) cells and CD4⁺CD25⁺Foxp3⁺ Tregs in tolerance induction by IL-2/IL-2mAb complexes treatment

To assess the FVIII-specific proliferative activity of Teff cells after IL-2/IL-2mAb complexes treatment, CD4⁺ T cells were isolated from spleens of three groups of mice including naive, isotype control mAb (IgG2a) + FVIII treated and IL-2/IL-2mAb complexes

+ FVIII treated mice 35 days after first FVIII protein injection. When stimulated with FVIII protein, CD4⁺ T cells isolated from IgG2a + FVIII treated mice (with high-titer anti-FVIII inhibitory antibodies) proliferated robustly on day 35 (Fig. 3a) after first FVIII protein injection. In contrast, CD4⁺ T cells isolated from IL-2/IL-2mAb complexes + FVIII treated mice showed no FVIII-specific proliferation (Fig. 3a); comparable levels of non-specific proliferation were observed between the cells with and without FVIII stimulation. No increase in proliferative responses to FVIII was also observed from CD4⁺ T cells isolated from control naive mice. Next, we tested the suppressive function of Tregs in tolerized mice treated with IL-2/IL-2mAb complexes + FVIII. The suppressive activity of CD4⁺CD25⁺ Tregs isolated from tolerized mice at 3 weeks following first FVIII injection were evaluated in a FVIII-specific suppression assay using CD4⁺ T cells from FVIII protein only treated mice as responder T (Tresp) cells. As anticipated, we observed significant FVIII-specific suppression by CD4⁺CD25⁺ Tregs on day 21 isolated from IL-2/IL-2mAb complexes + FVIII tolerized mice (Fig. 3b). Since TGF- β is critical for Tregs development, we also investigated the TGF- β 1 levels in the mouse plasma. The IL-2/IL-2mAb complexes + FVIII tolerized mice have increased TGF- β 1 levels at weeks 4, 4.5 and 5 after the first FVIII protein injection, compared to FVIII only treated and naive mice (Fig. 3c).

Un-related T-dependent antigen (TNP-KLH) challenge

Since IL-2/IL-2mAb complexes treatment temporally increased a large number of Tregs, the long-term effect on the immune system was investigated. Three IL-2/IL-2mAb complexes + FVIII tolerized mice and three FVIII only treated mice were inoculated with the T-dependent antigen, TNP-KLH, at 18 weeks after all the treatment. Untreated naive hemophilia A mice (n=3/group) were used as control in the experiment. Peak antibody titers were observed at 3 weeks post primary immunization and 1 week post secondary TNP-KLH immunization. The levels of total IgG (Fig. 4a) and specific IgM (Fig. 4b) were equivalent between tolerized mice, FVIII only treated and naive mice following challenges with TNP-KLH. These data indicated that transient immunomodulation with IL-2/IL-2mAb complexes did not adversely affect immune responses to T-dependent neoantigen.

Depletion of Tregs abolished the preventive effect of the IL-2/IL-2mAb complexes therapy from antibody formation

In a separate experiment, three groups of hemophilia A mice were treated with IL-2/IL-2mAb complexes + FVIII (12U/kg/mouse, 3x/week), IL-2/IL-2mAb complexes + FVIII + anti-CD25 mAb (PC-61), and FVIII only for 4 weeks. Anti-CD25mAb was used to deplete CD4⁺CD25⁺Foxp3⁺ Tregs *in-vivo*. [18] Following the tolerance induction period, we challenged the mice further with 40U/kg of FVIII weekly for 3 weeks. As shown in the results, none of the six mice treated with IL-2/IL-2mAb complexes + FVIII (n=6, Fig. 5a, left panel) generated inhibitor titers during the entire experimental period. However, in both of the PC-61 treated mice and FVIII only control mice (Fig. 5a, middle and right panels), inhibitor titers began to appear by week 3. Flow cytometry analysis showed that PC-61 significantly depleted CD4⁺CTLA4⁺Foxp3⁺ Tregs (Fig. 5b and 5c) in treated mice. These results confirmed the significant involvement of Tregs in suppressing anti-FVIII immune responses.

Induction of long-term tolerance to FVIII

To investigate whether IL-2/IL-2mAb complexes treatment can induce long-term tolerance to FVIII, we evaluated a series of transient immunomodulation protocols as shown in Table 1. When mice were initially treated with high dose of FVIII (80U/kg/mouse) during the immunomodulation period, long-term tolerance was not achieved even with a longer modulation regimen. As shown in Supplemental 3, FVIII only (80U/kg/wk) treated mice started to produce anti-FVIII antibodies on week 3 (Supplemental 3c, right panel), whereas mice treated with IL-2/IL-2mAb complexes for 6 weeks and 80U/kg of FVIII weekly produced antibody titers on week 7 (Supplemental 3c, left panel). Similarly, with more frequent injections of FVIII (12U/kg/mouse, 3x/wk), long-term tolerance was not established in treated mice (Supplemental 3a & b). Thus, we avoid using high dose or frequent infusions of FVIII to reduce danger signals during tolerance induction. Subsequently, by using a modulation protocol of IL-2/IL-2mAb complexes 3x/wk + FVIII 20U/kg/mouse/wk for 8 weeks, followed by FVIII only infusions (FVIII 20U/kg/mouse/wk for another 10 weeks), 10 out of 11 treated mice did not produce any antibody titers during modulation and follow up period (Fig. 6b, left panel). These mice also maintained high FVIII activities in the plasma collected 15 minutes after weekly FVIII infusions. Only one of the treated mice produced inhibitor titers starting on week 15 (Fig. 6b, right panel). All the control FVIII only treated mice generated antibodies starting on week 5 and their FVIII activities gradually declined to undetectable levels (fig. 6c). In addition, FVIII only treated mice induced high levels of anti-FVIII IgG1 in the plasma on weeks 8 and 12 (Fig. 6d), whereas the IL-2/IL-2mAb complexes + FVIII treated mice did not produce anti-FVIII IgG1 on weeks 4, 8 and 12 in the plasma. These results indicate that antigen levels present during treatment period of transient immunomodulation protocols have significant impact on the success rate of long-term tolerance induction.

Similarly to the prevention experiments shown in Fig. 2, we have evaluated CD4⁺Foxp3⁺Helio⁺ Tregs over the tolerance induction period and examined their correlation with the FVIII activities/inhibitor titers at each time point. The CD4⁺Foxp3⁺Helio⁺Tregs were significantly expanded during the period of IL-2/IL-2mAb treatment, however, the levels gradually dropped to basal levels after treatment. In addition, plasma kynurenine levels were examined in each treated and naive mouse group. There were significant increases in kynurenine levels in mice receiving the IL-2/IL-2mAb complexes + FVIII compared with other control groups (Fig. 6e). The levels were concomitant with Treg expansion during the modulation period in the treated mice, and remained slightly elevated at the end of the 18 weeks follow up period.

DISCUSSION

Immune response against FVIII is a major obstacle for protein replacement therapy in hemophilia A treatment. Our lab has demonstrated that a single cycle injection of the particular IL-2/IL-2mAb complexes completely prevented the formation of anti-FVIII antibodies in hemophilia A mice following FVIII plasmid-mediated gene therapy[17]. However, the single cycle IL-2/IL-2mAb complexes treatment was unsuccessful to prevent antibody production in mice treated with protein replacement therapy probably due to more

robust immune responses and danger signals generated from frequent protein infusions (data not shown). Thus, we investigated if multiple cycles of IL-2/IL-2mAb complexes treatment is effective. We found that treatment with 4 cycles of IL-2/IL-2mAb complexes and FVIII protein (80U/kg/mouse/week) for 4 weeks prevented anti-FVIII antibody production in all treated mice without further challenges with FVIII protein. Similarly to our previous findings[17], IL-2/IL-2mAb complexes induced high levels of Tregs (5–7-fold) as well as increased the expression levels of their activation markers, CD25, CTLA4 and GITR. In addition, treated mice had increased levels of immune suppressive cytokine TGF- β 1 in plasma, which correlated with the expansion and activation of Tregs. It is also found that the expanded Tregs are predominantly thymus derived Helios⁺ natural Tregs which possess more stable and higher suppressive function compared with peripherally induced Tregs[20]. Indeed, an *in vitro* suppressive assay confirmed the strong suppressive activity of the expanded Tregs towards FVIII-specific immune responses. Furthermore, a specific anti-CD25 antibody (PC-61) depleted the activated Tregs and blocked the preventive effect of IL-2/IL-2mAb complexes from antibody production in hemophilia A mice. These results together indicated that *in vivo* expansion of Tregs by IL-2/IL-2mAb complexes modulated and prevented the anti-FVIII antibody responses in FVIII protein treated hemophilia A mice.

Many successful protocols[18, 21–23] to modulate FVIII-specific immune responses involve increases in the percentages and/or total numbers of CD4⁺Foxp3⁺Tregs in either protein replacement or gene therapy settings. Importantly, these induced Tregs must be activated to exert their regulatory function. Recently Moghimi et al.[24] developed a prophylactic immune tolerance protocol to FVIII using rapamycin in hemophilia A mice; this protocol effectively prevented formation of inhibitors to FVIII by an increase in the frequency of CD4⁺CD25⁺Foxp3⁺ Tregs through suppressed Teff (the Th2) responses in the tolerized mice. In our experiments, IL-2/IL-2mAb complexes induced 5–7 fold expansion of thymus-derived natural Tregs; these short-lived, expanded Tregs were highly activated and displayed superior suppressive function. The IL-2/IL-2mAb complexes protocol is believed to be a more potent and efficient strategy with increase in actual numbers rather than percentages of FVIII-specific Tregs to modulate anti-FVIII immune responses. In addition, our results directly demonstrate the important role of Tregs in suppressing anti-FVIII immune responses.

Furthermore, IL-2/IL-2mAb complexes treatment is considered to be relatively safer compared to many other immunomodulation agents. Recent clinical trials using low dose of IL-2 to promote increased function of Tregs showed little side effects[25, 26]. We found that IL-2/IL-2mAb complexes selectively and specifically expanded Tregs without deleterious effect to induce expansion of other cell populations including CD4⁺ and CD8⁺ T, B220⁺ B, and CD11b⁺ myeloid cells[17]. We also demonstrated that transient expansion of Tregs only resulted in temporary suppression of immune responses. When FVIII tolerized mice were subsequently challenged with an unrelated antigen (TNP-KLH), the mice mounted normal immune responses, indicating that transient immunomodulation did not hamper immune competence in these animals. Upon cell-cell contact, Tregs inhibit TCR-induced proliferation[27]. This direct suppression was via the immunosuppressive cytokines such as TGF- β [28], IL-35[29] and IL-10 [30] which were secreted by Tregs. Additionally, Tregs

suppress specific types of immune responses via a series of transcriptional events mediated by Foxp3 and corresponding transcription factors such as interferon regulatory factor-4 (IRF-4)[31] in target Th2 cells. It is hypothesized that different mechanisms may operate depending on the site of immune reaction, the type and activation state of the target cell as well as the activation status of Tregs.

Although we successfully prevented antibody formation in FVIII protein treated hemophilia A mice, it was very difficult to induce long-term tolerance by continuous challenge of FVIII protein. We attempted a series of experiments with different treatment schedules of IL-2/IL-2mAb complexes and different dosages of FVIII protein (Table 1). We found that high doses or frequent infusions of FVIII should be avoided to reduce danger signals during the initial phase of tolerance induction. Our successful protocol to induce long-term tolerance used low dose FVIII 20U/kg/mouse/week in combination with IL-2/IL-2mAb complexes treatment for 8 weeks, followed by FVIII only (20U/kg/mouse/week) for another 10 weeks. The treated mice maintained FVIII-specific tolerance without producing inhibitors. Higher dose of FVIII can probably stimulate more effector T cells, which may not be completely suppressed by the initially expanded Tregs. However, lower dose of FVIII (20U/kg FVIII which is close to clinical human FVIII dosage) is sufficient for the tolerance induction phase. Furthermore, once the tolerance is induced and associated with established FVIII-specific Tregs, higher dose of FVIII (40–80U/kg) can be used for treatment without inducing antibody responses (data not shown). Based on these results, both FVIII dosages and IL-2/IL-2mAb complexes treatment duration are very important factors to successfully induce long-term tolerance.

In the tolerance induction experiments, we also examined the changes in Tregs over 18 weeks. We found that similarly to the prevention experiments, IL-2/IL-2mAb complexes induced significant expansion of Tregs in the treatment period, however the levels gradually returned to basal levels comparable to those in naive mice. It is believed that FVIII-specific CD4⁺Foxp3⁺Tregs were generated during the modulation period and recruited to the secondary lymphoid organs to maintain tolerance. This interesting hypothesis needs future investigation. Previously, Fletcher's group reported that Indoleamine 2,3-dioxygenase (IDO) attenuated inhibitor development in gene-therapy-treated hemophilia A mice[32]. IDO is a potent immunosuppressive enzyme that can inhibit T-cell responses and induce T-cell apoptosis by regulation of tryptophan metabolism. The metabolites of tryptophan, especially kynurenines, have been shown to have strong immune regulatory properties. We found both TGF- β and kynurenine levels were elevated in mouse plasma synchronized with Treg expansion in the IL-2/IL-2mAb complexes + FVIII treated mice, suggesting that the modulation effects were correlated with the activation of IDO-system. TGF- β and kynurenine were recently found to be the key components to promote infectious tolerance[33]. It is interesting that we have previously observed induction of infectious tolerance after adoptive transfer of FVIII-specific hemophilia A/Foxp3 transgenic Tregs in the recipient HemA mice[21]. The concomitant increases in the levels of TGF- β and kynurenine during Treg expansion following IL-2/IL-2mAb complexes treatment induced a positive feedback loop for a suppressive phenotype to promote generation of antigen-specific Tregs, apoptosis of effector T cells, and suppression of activated T cells, leading to induction of antigen-specific tolerance.

In conclusion, we took the advantage of using IL-2/IL-2mAb complexes to expanded Tregs *in vivo* and continuous treatment with low dosages of FVIII protein to achieve induction of long-term tolerance to FVIII in hemophilia A mice in a short period of time. IL-2/IL-2mAb complexes treatment is a safe and effective immunomodulation agent that has potential to translate from bench to bedside for long-term tolerance induction to FVIII in the treatment of hemophilia A. It is also important to point out the potential of this strategy to be used in combination protocols for treating pre-existing/refractory anti-FVIII immune responses in place of the very expensive and lengthy ITI protocol. The successful strategy using IL-2/IL-2mAb complexes and continuous treatment of appropriate dosages of antigen also has important implications for treating other immune related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Immunomodulation with IL-2/IL-2mAb complexes to prevent antibody production in hemophilia A mice following FVIII protein replacement therapy

The immunomodulation schedule was shown in (a). (b) Anti-FVIII antibody titers over time. Hemophilia A mice ($n=7$; left panel) were treated with daily injection of IL-2/IL-2mAb complexes three times per week plus FVIII 80U/kg/mouse/week for four weeks. The control group ($n=3$; right panel) received 80U/kg/mouse FVIII only. Antibody titers were then evaluated weekly by Bethesda assay from week 4 to 12. Each symbol represents data obtained from an individual mouse. (c) Serum anti-FVIII IgG1 levels were evaluated for the treated and naive mouse groups by ELISA at weeks 4, 5 and 6 after the treatment period. Data shown is representative of two independent experiments.

Figure 2. Effect of IL-2/IL-2mAb complexes treated mice on CD4⁺ T cells, CD4⁺CD25⁺FoxP3⁺ Tregs and CD4⁺Foxp3⁺Helios⁺ T cells in periphery blood

Blood cells were isolated from Naïve, FVIII only and IL-2/IL-2mAb complexes + FVIII treated mice. Cells were stained and analyzed for (a) CD4⁺ T cells, (b) CD4⁺CD25⁺FoxP3⁺ T cells and (c) CD4⁺Foxp3⁺Helios⁺ T cells populations by flow cytometry. Left panels show the representative dot plots on the peak day of Treg expansion and the numbers in the figures denote the percentages of corresponding T-cell populations; right panels show the changes in these cell populations over time. (d) The median fluorescence intensity (MFI) value of the three activation markers of Tregs: CD25, GITR and CTLA-4 at serial time points from each treated mouse group by flow cytometry analysis (means $\pm$ SEM values obtained from each mouse group). Values of p were as follows: **, <0.005; *, <0.05. Data shown is representative of two independent experiments.

Figure 3. Functional examination of CD4⁺ T cells and CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells isolated from mice treated with IL-2/IL-2mAb complexes by proliferation, suppression and cytokine expression assays *in-vitro*

(a) Proliferation assay. Splenic CD4⁺ T cells from naïve, IgG2a + FVIII and IL-2/IL-2mAb complexes + FVIII treated mice (n=2/group at each time point) 35 days after FVIII injection were stimulated with 80U/kg FVIII for 72 hours, and followed by [³H] thymidine incorporation. (b) FVIII-specific suppressive assay. T-responder (Tresp) cells were isolated from spleens of inhibitor mice treated with FVIII protein. CD4⁺CD25⁺ T cells from pooled splenic cells of mice at day 21 following FVIII + IgG2a and FVIII + IL-2/IL-2mAb complexes treatment were used as suppressive cells. The final coculture system consisted of CD4⁺ Tresp cells, APCs, and Tregs at the indicated Treg : Tresp ratio. Data shown are mean $\pm$ SD of counts per minute (c.p.m.) of [³H] thymidine incorporation in triplicate wells. (c) Systemic TGF- β 1 levels. Systemic TGF- β 1 in the plasma of naïve, FVIII only and IL-2/IL-2mAb complexes + FVIII treated mice (n=3/group) were evaluated by ELISA assay at week 4, 4.5 and 5 after the first FVIII injection. Data of mean $\pm$ SD of each mouse group are shown.

Figure 4. Challenge of tolerized mice with unrelated T-dependent antigen

Tolerized mice (mice treated with FVIII + IL-2/IL-2mAb complexes, n=2/group), mice treated with FVIII only (n=2/group) and naive mice (n=2/group) were challenged with T-dependent antigen (TNP-KLH, 100ug/mouse) starting at week 22 after first FVIII injection. (a) Anti-TNP IgG and (b) Anti-TNP IgM levels were assessed over time following primary (day 0) and secondary (day 21) immunization. Data shown is representative of three independent experiments.

Figure 5. Blockade of the protective effect from anti-FVIII antibody production by administering an anti-CD25 mAb in IL-2/IL-2mAb complexes + FVIII treated hemophilia A mice

In the 4 weeks immunomodulation period, mice were treated with IL-2/IL-2mAb complexes + FVIII (12U/kg $\times$ 3/week), IL-2/IL-2mAb complexes + anti-CD25 (PC-61) + FVIII (12U/kg $\times$ 3/week) and FVIII only control. Subsequently, mice were treated with FVIII (40U/kg/week) for additional 3 weeks. (a) Anti-FVIII inhibitor titers (BU) for each treated group: Left panel, IL-2/IL-2mAb complexes + FVIII; middle panel, IL-2/IL-2mAb complexes + FVIII + PC-61; right panel, FVIII only. Data represented inhibitor titers of each individual mouse. (b) Blood cells were isolated from IL-2/IL-2mAb complexes + FVIII, IL-2/IL-2mAb complexes + PC-61 + FVIII, FVIII only and naive mice (n=4/group), stained and analyzed for CD4⁺Foxp3⁺CTLA4⁺ T cells by flow cytometry over time. (c) The MFI values of the three activation markers on Tregs from each treated mouse group. Data shown is representative of two independent experiments.

Figure 6. Induction of long-term tolerance to FVIII by *in vivo* expansion of Tregs in hemophilia A mice

The immunomodulation treatment schedule was shown in (a). (b) Hemophilia A mice (n=11) were treated with IL-2/IL-2mAb complexes + FVIII for eight weeks. The mice were subsequently treated with FVIII only for additional 10 weeks. (c) The control group (n=3) received FVIII only for 18 weeks. FVIII activities (b and c, left panels) and Anti-FVIII antibody titers (b and c, right panels) were performed using blood samples. Each symbol represents data obtained from an individual mouse. (d) Serum anti-FVIII IgG1 levels and (e) Plasma kynurenine levels for the treated mice were evaluated during and after the 8 weeks immunomodulation period. Naive and FVIII only treated mice were used as controls. Data shown is representative of two independent experiments.

Table 1

Dosages and schedules used in tolerance induction protocols

FVIII			IL-2/IL-2mAb complexes		
Dosage	Treatment (wks)	Tolerance induction and maintenance period	Dosage (6 µg/mouse)	Treatment (wks)	
120U/kg/mouse (3×/wk)	2	-	3×/wk	2	
	2	40U/kg/wk	3×/wk	2	
80U/kg/mouse (once/wk)	4	-	3×/wk	1,2 or 4	
	4	80U/kg/wk	3×/wk	4 or 6	
12U/kg/mouse (3×/wk)	4	40U/kg/wk	3×/wk	4 or 6	
	4	40U/kg/wk	6×/wk	4	
20U/kg/mouse (once/wk)	4	20U/kg/wk	3×/wk	6 or 8	