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ARTICLE

Ex vivo expanded autologous polyclonal regulatory T cells suppress inhibitor formation in hemophilia

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Adoptive cell therapy utilizing *ex vivo* expanded polyclonal $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) is in use in clinical trials for the treatment of type 1 diabetes and prevention of graft versus host disease in bone marrow transplantation. Here, we seek to evaluate this approach in the treatment of inherited protein deficiencies, *i.e.*, hemophilia, which is often complicated by antibody formation against the therapeutic protein. Treg from mice that express green fluorescent protein–marked FoxP3 were highly purified by two-step magnetic/flow sorting and *ex vivo* expanded 50- to 100-fold over a 2-week culture period upon stimulation with antibody-coated microbeads. FoxP3 expression was maintained in >80% of expanded Treg, which also expressed high levels of CD62L and CTLA-4. Transplanted Treg suppressed inhibitory antibody formation against coagulation factors VIII and IX in protein and gene therapies in strain-matched hemophilia A and B mice, including in mice with pre-existing antibodies. Although transplanted Treg became undetectable within 2 weeks, suppression persisted for >2 months. Additional studies suggested that antigen-specific suppression emerged due to induction of endogenous Treg. The outcomes of these studies support the concept that cell therapy with *ex vivo* expanded autologous Treg can be used successfully to minimize immune responses in gene and protein replacement therapies.

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INTRODUCTION

In the mammalian immune system, lymphocytes play a crucial role in the generation of antigen-specific immunity against tumors and invading pathogens. As a consequence of this exquisite specificity and protection, deleterious and unwanted immune responses to self-antigens and foreign proteins must be controlled. To counteract these unwanted immune responses, a population of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) emerges during thymic development ("natural" or "nTreg") or is induced peripherally ("iTreg") in a transforming growth factor-β dependent process.[1] These $CD4^+$ Treg constitutively express the transcription factor FoxP3 and CD25 (the α chain of the interleukin (IL)-2 receptor), are required to prevent autoimmune disease, and are critical regulators of responses to non-self antigens (*e.g.*, food and environmental antigens). Consequently, induction and recruitment of Treg is a major target in several medical interventions such as organ transplantation or treatment of autoimmune disease.[2,3] Recent studies have shown that this cell type is also critical in the response to therapeutic proteins introduced via protein or gene replacement therapy for genetic diseases.[4] For example, Treg induction is a major mechanism by which immune tolerance to transgene products is induced following hepatic gene transfer with adeno-associated viral (AAV) or lentiviral vectors or following transplant of gene modified B cells.[5–7] Treg suppress effector T cells and other immune cell types via a variety of mechanisms. These are typically cell contact dependent *in vitro* but may involve secretion of cytokines and other immune suppressive molecules *in vivo*.[1] Technologies have been established to expand Treg *ex vivo* for the development of tolerogenic cell therapies. Expansion of polyclonal Treg (derived from peripheral blood or cord blood) is in clinical development for the treatment of Type 1 diabetes (T1D) and for prevention of graft versus host disease (GvHD) in bone marrow and hematopoietic stem cell transplantation.[8–11] A different subset of regulatory $CD4^+$ T cells, T regulatory Type 1 or Tr1 cells, is in clinical trial for Crohn's disease (an inflammatory bowel disease) and for prevention of GvHD in hematopoietic stem cell transplant.[12,13] Tr1 cells express large amounts of the suppressive cytokine IL-10 and have recently been defined as $CD4^+CD49b^+LAG\text{-}3^+$ T cells.[14]

Treg have several advantages compared to other immune modulatory drugs, including a natural immune regulatory ability, avoidance of severe side effects and global immune suppression typically associated with conventional drugs, reduced risk of long-term damage to the immune system, and potential for a lasting tolerogenic response. We sought to investigate whether this promising new tolerogenic

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cell therapy, based on administration of *ex vivo* expanded Treg, could be used in treatment of genetic disease. As an example, we chose gene and protein replacement therapy for the X-linked bleeding disorder hemophilia. Hemophilia A and B result from deficiency of clotting factor VIII (FVIII) or IX (FIX), respectively. The disease affects 1 in 5,000 male births for hemophilia A and 1 in 30,000 for hemophilia B worldwide.[15,16] An adaptive immune response (formation of inhibitory antibodies) to the therapeutic protein denotes a serious complication of treatment. Inhibitor formation occurs in 20–30% of severe hemophilia A and ~5% of severe hemophilia B patients (those with <1% residual coagulation activity), complicates treatment, and raises risks for morbidity and mortality. Inhibitors typically develop in early childhood and are dependent on help by $CD4^+$ T cells. Risk factors include the underlying mutation, intensity of early treatment, polymorphisms in several genes affecting immune functions, and likely also human leukocyte antigen (HLA) genes.[17–20] Immune tolerance induction protocols for elimination of inhibitors are based on daily high-dose infusion of factor concentrate. This regimen is lengthy (months to >1 year), expensive, and not successful in all patients. Preclinical studies in various animal models indicate that the risk of inhibitor formation in gene therapy depends on the underlying mutation and the specific gene transfer protocol, including choice and design of vector, dose, and route of administration.[21]

In the following, we demonstrate in three different experimental settings (FVIII protein therapy in naive or preimmune hemophilia A mice and muscle-directed FIX gene transfer in hemophilia B mice) that administration of *ex vivo* expanded autologous $CD4^+CD25^+FoxP3^+$ Treg at doses similar to those currently used in clinical trials (~5×10^7 cells/kg) can substantially suppress inhibitor formation despite limited persistence of the transplanted cells.[22] Mechanistically, we provide evidence that transplanted Treg facilitate the induction of antigen-specific Treg, a mechanism also referred to in the literature as "infectious tolerance."[23] We propose that this cell therapy could be incorporated into several different treatment protocols for hemophilia and other genetic diseases to reduce the risk of deleterious immune responses.

RESULTS

Robust *ex vivo* expansion of murine $FoxP3^+$ Treg cells

In contrast to the advanced protocols and reagents available for the *in vitro* expansion of human Treg, such approaches for the expansion of high purity murine Treg (to avoid contamination and outgrowth of effector T cells) are lacking. To develop a murine model of Treg therapy we used a BALB/c strain with a green fluorescent protein (GFP) reporter, expressed in conjunction with transcription factor FoxP3 (FoxP3-IRES-GFP^+ BALB/c), to facilitate enrichment of a highly pure Treg population. Indeed, magnetic pre-enrichment for splenic $CD4^+$ T cells from these FoxP3-IRES-GFP^+ BALB/c mice followed by flow cytometry sorting of GFP^+ ($FoxP3^+$) cells resulted in Treg preparations with 95–99% purity upon post-sort analysis (Figure 1a). We were able to expand these Treg 7- to 10-fold after a first stimulation with anti-CD3/CD28 coated microbeads, with a second restimulation yielding an overall 50- to 100-fold expansion (Figure 1b). The second stimulation with anti-CD3/-CD28 beads was typically performed on day 7, but was varied by ±1 day depending on cell size and activation state of Treg. Proliferating cells typically assumed an irregular morphology and were larger in size. Cells were restimulated once they returned to their prestimulation size and when proliferation caused a substantial increase in the ratio of cells to beads.

Conditioned media contained high levels of IL-2 (2,000 IU/ml), which was replenished every other day, assuming consumption. Similar proliferation levels were obtained on addition of either mouse or human IL-2 or when serum-free media were compared to media containing 10% fetal bovine serum (data not shown). We found maximal expansion of cultured Treg on day 14, but also observed a loss in GFP expression in 10–30% of cells at this late

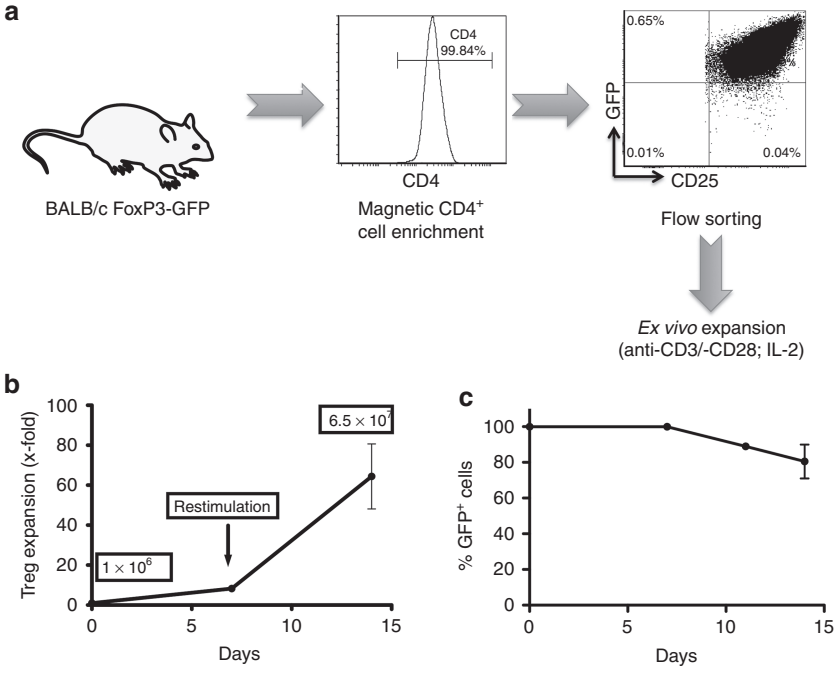


Figure 1 Isolation and *ex vivo* expansion of murine $CD4^+CD25^+FoxP3^+$ Treg. (**a**) Splenocytes are isolated from BALB/c mice with a GFP reporter gene knock-in at FoxP3 locus. GFP^+ cells are sorted to 95–98% purity from magnetically purified $CD4^+$ splenocytes. (**b**) Expansion of Treg (starting with 1×10^6 cells) after two rounds of stimulation with anti-CD3/-CD28 beads. Data are average ±SD from 5 independent experiments. (**c**) Percent GFP^+ (*i.e.*, FoxP3 expressing) cells as a function of days in culture.

time point (Figure 1c). However, no contaminating CD8$^+$ T cells were found (data not shown). We determined that Treg on day 11 of culture had stable expression of GFP (80–90%) while still showing a high expansion profile (~50-fold). For all *in vivo* studies detailed below, only Treg preparations with >80% GFP expression were used.

Ex vivo expanded Treg persist only transiently *in vivo*

The distribution of *ex vivo* expanded Treg into different immune compartments in the host was quantified at 24 hours after tail-vein injection. Expanded GFP$^+$ Treg engrafted at higher frequencies in immune compartments of recipient mice (15–40%), as compared to mice that received freshly isolated Treg (1–3%, Figure 2a). Total numbers of GFP$^+$ Treg were substantially lower in nonimmune compartments (lung and the liver). In order to evaluate persistence of *ex vivo* expanded Treg, we quantified the frequencies of expanded donor thy1.2 GFP$^+$ Treg in the blood and spleen of recipient thy1.1 BALB/c mice (Figure 2b). *Ex vivo* expanded Treg (1×10^6) from thy1.2 mice were infused into thy1.1 BALB/c recipient animals by tail-vein injection. Donor Treg were detected at frequencies of 18–23% of total Treg in peripheral blood and spleens of recipients at day 2, but declined to 2–3% on day 7 and to undetectable levels on days 14 and 21. Adoptively transferred Treg were thy1.2$^+$GFP$^+$ (*i.e.*, FoxP3$^+$) at all time points, without evidence for conversion to thy1.2$^+$GFP- (*i.e.*, FoxP3$^-$) effector CD4$^+$ T cells. Prior studies also support the interpretation that transferred Treg do not persist at a detectable level in the host.[8,24] To ascertain if the rapid decline in GFP$^+$ Treg in the host was due to an immune response to GFP, a GFP-specific MHC I pentamer was used to determine CD8$^+$ T cell responses to injected Treg (Figure 2c). There was no evidence for the development of CD8$^+$ CTL responses to injected donor GFP$^+$ Treg, whereas another group of recipient mice readily developed a CD8$^+$ CTL response to a GFP expressing adenovirus, which was used as a positive control (Figure 2c).

Expanded Treg maintain suppressive phenotype and can induce tolerogenic dendritic cells *in vitro*

After 11 days in expansion culture, Treg were similar to freshly isolated Treg for most phenotypic markers. The expression of Treg associated markers CD4, CD25, FoxP3, cytoplasmic CTLA-4 (CD152), the lymphoid homing receptor CD62L, and the Ikaros family transcription factor helios was determined by flow cytometry (Figure 3a). *Ex vivo* expanded Treg were CD4$^+$CD25$^+$, and 80–90% of cells expressed FoxP3 (Figure 3a). As expected, a strict correlation between FoxP3 and GFP expression was observed. Compared to freshly isolated Treg, an increase in intracellular CTLA-4 (to ~98%) was observed in cultured Treg. Expanded cells on day 11 of culture were mostly CD62L$^+$ (~90%), while a reduction in CD62L expression

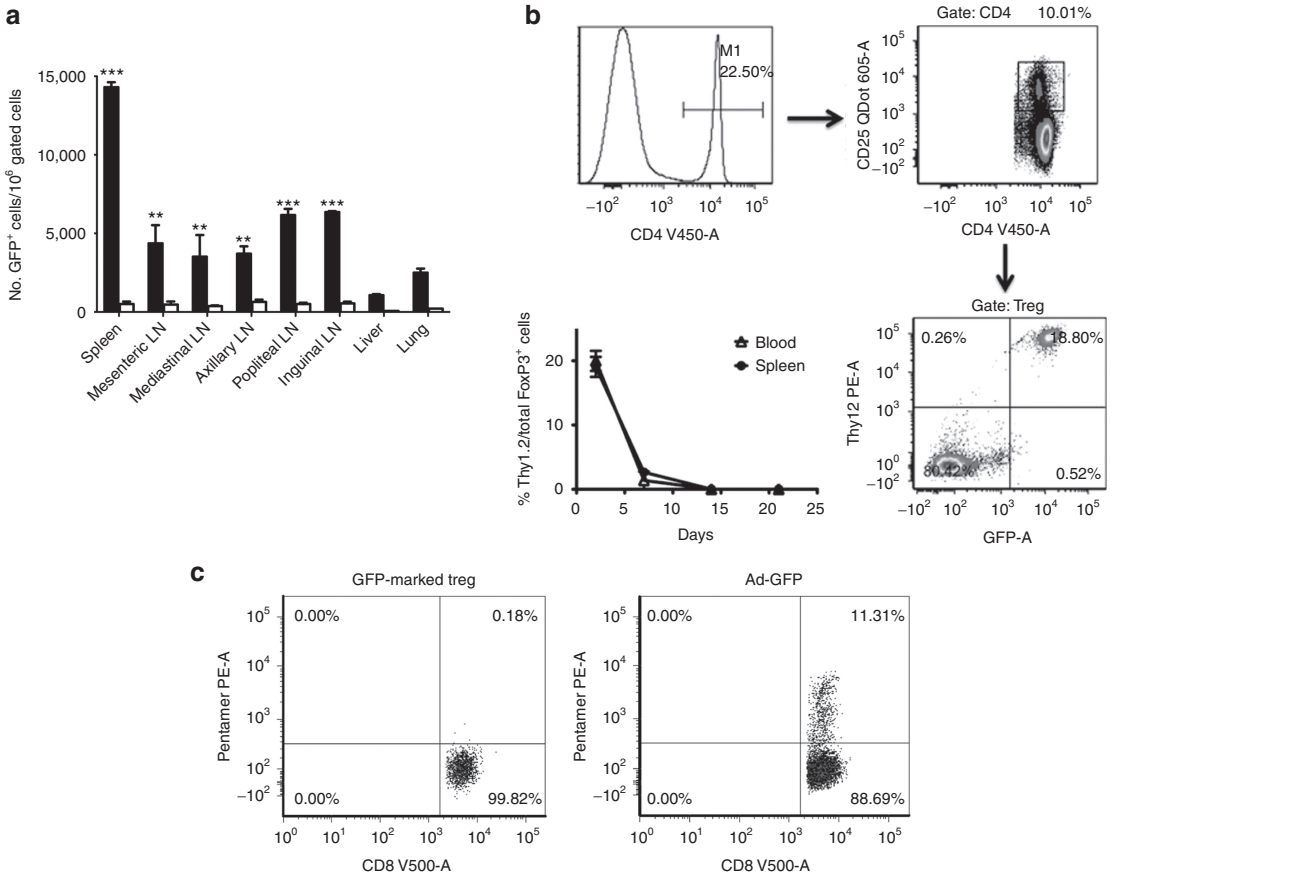
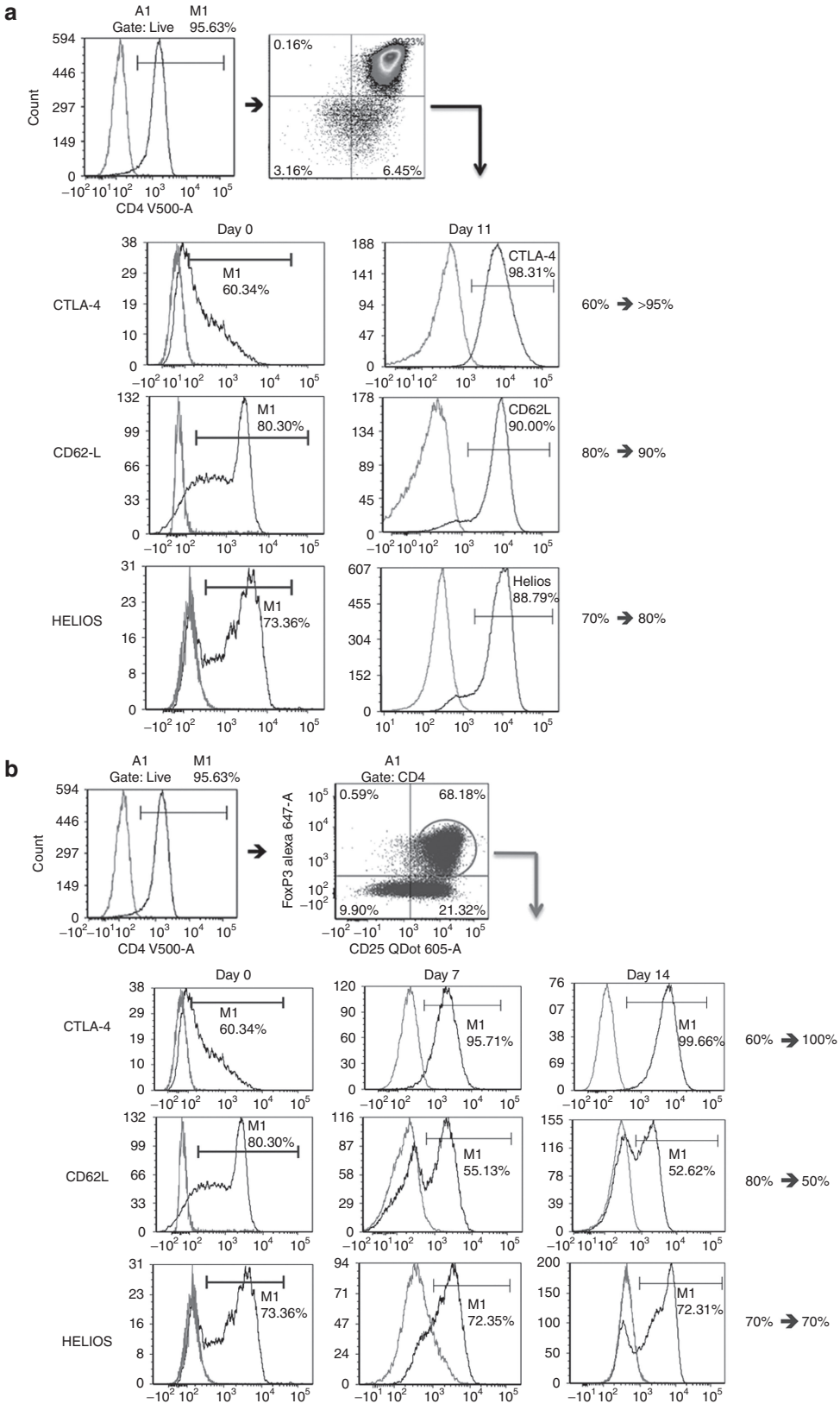


Figure 2 Distribution and enumeration of expanded Treg in recipient mice. (**a**) *Ex vivo* expanded Treg (dark bars) or freshly isolated Treg (white bars) were administered into recipient mice by tail-vein injection. Twenty-four hours later, spleen, liver, lung, and lymph nodes were harvested from recipient animals to determine frequencies of CD4$^+$CD25$^+$GFP$^+$ donor Treg. Values are denoted as number of donor Treg per 1×10^6 live gated cells. (**b**) Frequency of expanded thy1.2$^+$GFP$^+$ Treg (11 days of culture) in peripheral blood and spleen as a function of time after intravenous administration of 1×10^6 cells to thy1.1 expressing BALB/c mice. Data are average ±SD ($n = 4$ per time point) of percent thy1.2$^+$ cells of CD4$^+$FoxP3$^+$ T cells. Insert shows example of analysis by flow cytometry, indicating that injected thy1.2$^+$ remained GFP$^+$ (*i.e.*, continued to express FoxP3). (**c**) Determination of CD8$^+$ T cell response to GFP$^+$ donor cells using a GFP specific MHC I pentamer. Blood samples were analyzed on day 10 following IV injection of GFP$^+$ Treg or intramuscular injection of AD5-GFP. Plots are representative of data from three animals.

to ~50% was observed on day 14 (Figure 3b).[25] Helios expression (70–80%) was not significantly different from freshly isolated Treg for either time point. Expanded cells were also found to be GITR (glucocorticoid-induced TNFR family related gene)$^+$ and CD127^{lo} (data not shown).

We tested the capacity of *ex vivo* expanded Treg to suppress CD3/CD28 stimulated allogeneic CD4$^+$ T cells *in vitro*. Dilution of CellTrace violet in labeled responder CD4$^+$ T cells was used as an indicator of proliferation. Expanded Treg strongly suppressed the proliferative response of allogeneic CD4$^+$ T cells at 1:1 and 1:4 ratios of Treg to



responders when cocultured with CD3/CD28 beads for 72 h (Figure 3c), while maximal proliferation was observed when responder $CD4^+$ T cells were stimulated in the absence of Treg. In order to define a mechanism for suppression, bone marrow derived dendritic cells (DC) and splenic DC were primed with freshly isolated Treg, expanded Treg or Teff cells that were $CD4^+CD25^-$. In comparison to DC primed with freshly isolated Treg or Teff, DC that were primed with expanded Treg showed an immature phenotype, with a decrease in expression of CD80 and CD86 costimulatory molecules (Figure 3d). These results were reproducible in a second experiment (data not shown).

Ex vivo expanded polyclonal Treg suppress inhibitor formation in FVIII replacement therapy in naive or immune hemophilia A mice

Next, we evaluated the functional capacity of the expanded Treg to suppress inhibitor formation in protein replacement therapy of FVIII. Expanded Treg were intravenously injected at a dose of 1×10^6 into hemophilia A mice (BALB/c F8e16$^{-/-}$, $n = 6$) followed with 8 weekly injections of 1 IU of B domain-deleted recombinant human FVIII (BDD-FVIII; Figure 4a). Inhibitor formation was undetectable at 1 month of treatment and remained very low (1–4 BU) at 2 months in mice that received Treg (Figure 4b), while control mice ($n = 6$, no Treg therapy) developed high-titer inhibitors (on average 36 BU/ml by 2 months). Total anti-FVIII IgG titers, as measured by ELISA, were also significantly suppressed, albeit not as strongly by 2 months (Figure 4c). Consistent with data presented above, administered Treg had become undetectable in peripheral blood within 1 month (data not shown). In a repeat experiment, Treg-treated mice showed again significant suppression of inhibitor formation to BDD-FVIII (n = 7–9/experimental group, Figure 4e). However, repeated Treg dosing (three doses, 1–2 weeks apart, $n = 5$) suppressed inhibitor titers to undetectable levels (Figure 4d,e). These differences in inhibitor formation were also reflected in anti-FVIII IgG1 titers (Figure 4f).

Subsequently, we wanted to test the performance of expanded Treg in mice with ongoing immune response to FVIII. After 2 months of therapy with BDD-FVIII, hemophilia A mice developed inhibitors. A cohort of 12 mice with very similar inhibitor titers (~40 BU/ml) was identified, and half of these animals received 1×10^6 Treg each. FVIII replacement therapy was continued for 2 more months in all animals (Figure 5a). Treg treated mice showed no further increase in inhibitor or IgG titers against FVIII, while control mice showed a substantial further rise to >100 BU/ml, corresponding to a similar increase in anti-FVIII IgG titers (Figure 5b,c).

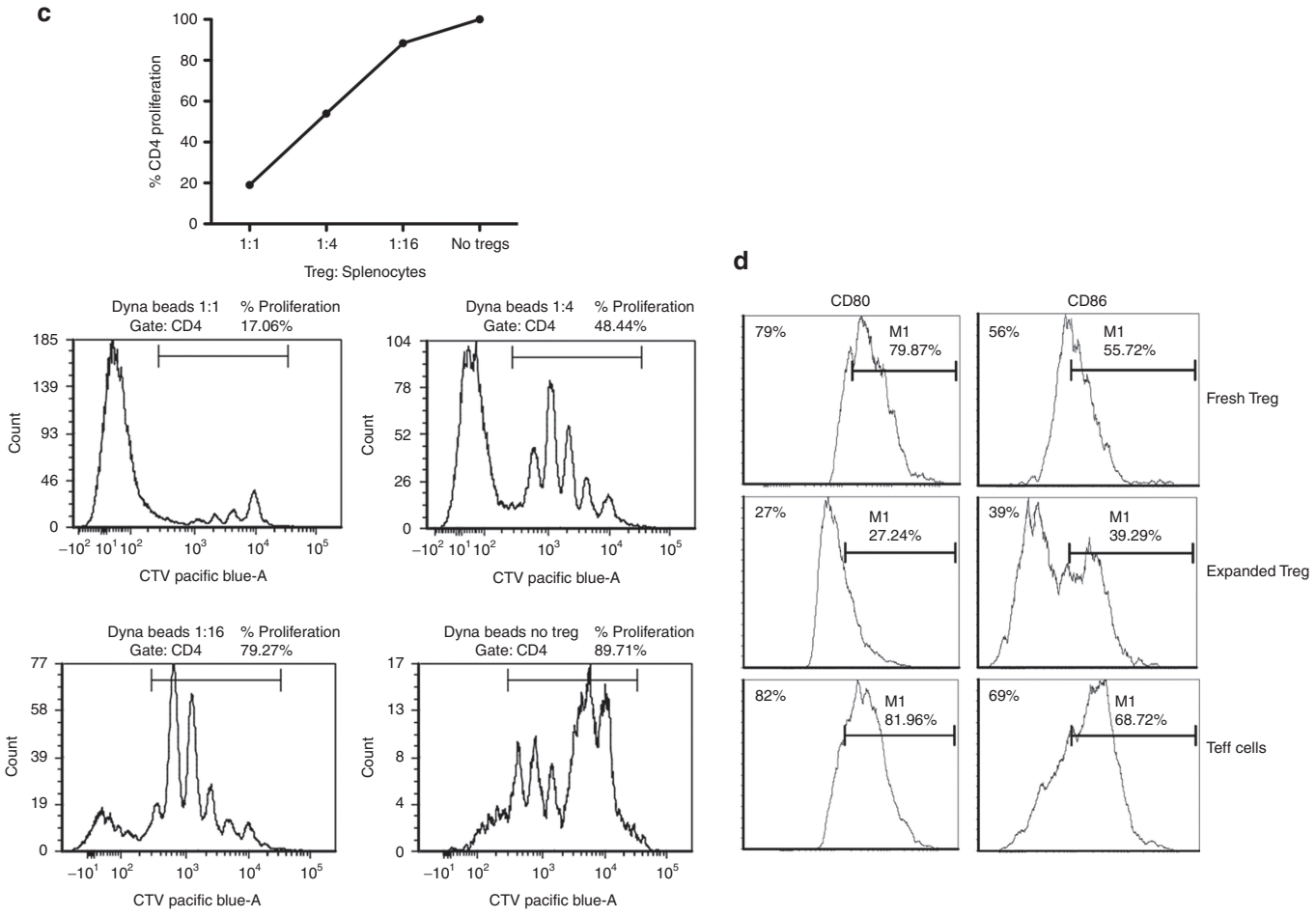


Figure 3 Phenotype and *in vivo* persistence of expanded Treg. (**a**) CTLA-4, CD62L, and helios expression in $CD4^+CD25^+FoxP3^+$ Treg (fresh after isolation or after 11 in culture; representative example, cells were stimulated with beads on days 0 and 7). (**b**) CTLA-4, CD62L, and helios expression in $CD4^+CD25^+FoxP3^+$ Treg at days 0, 7, and 14 in culture (representative example, cells were stimulated with beads on days 0 and 7; data for days 0 and 7 are prior to addition of beads). (**c**) *In vitro* suppression assay for expanded Treg demonstrating Treg dose-dependent suppression of anti-CD3/CD28 bead stimulated BALB/c splenocytes (CellTrace Violet dilution assay). (**d**) CD80 and CD86 expression in $CD11c^+$ DC primed for 3 days with either freshly isolated Treg, *ex vivo* expanded Treg, or $CD4^+CD25^-$ Teff cells. Data are percent DC that stained positive for CD80/86. Plots are representative of two independent experiments.

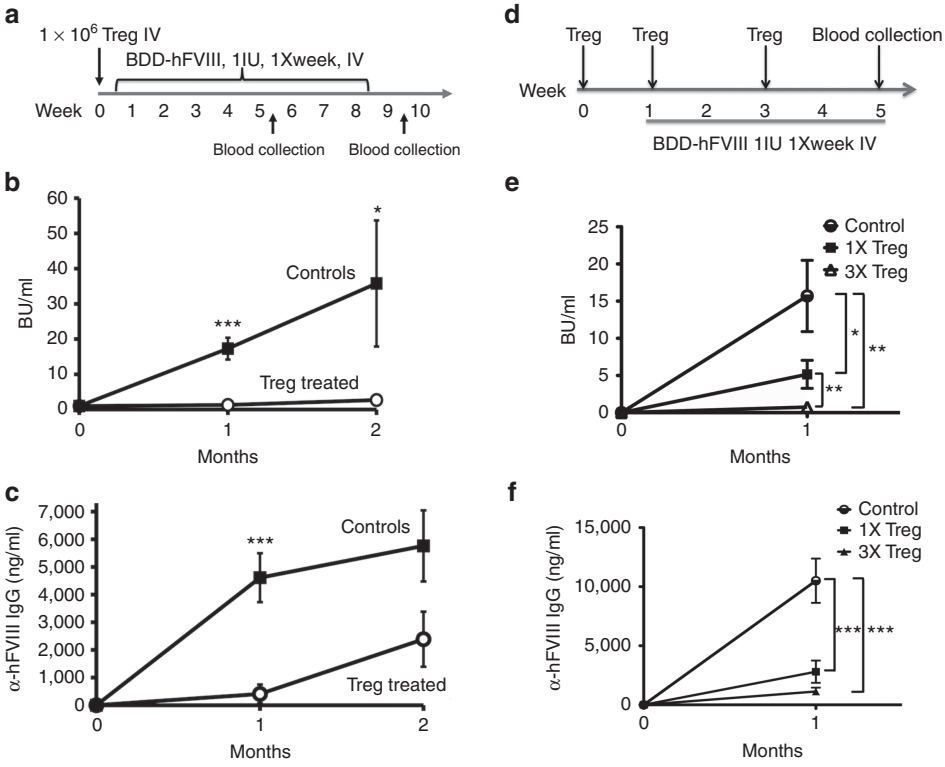


Figure 4 Expanded Treg suppress inhibitor formation against FVIII in hemophilia A mice receiving factor replacement therapy. (**a**) Experimental timeline. Hemophilia A mice (BALB/c *F8*e16$^{-/-}$) received an IV injection of 1×10^6 expanded Treg ("Treg treated"; whereas "control" mice did not receive cells). All animals ($n = 6$ per experimental group) subsequently received weekly IV injections of recombinant human B domain-deleted FVIII (BDD-FVIII, 1 IU per mouse and dose) for 2 months. (**b**) Inhibitor titers (BU/ml) as a function of time. (**c**) Anti-FVIII IgG titers (ng/ml) as a function of time. (**d**) Schematic timeline of mice that received three repeated doses of 1×10^6 expanded Treg ("3xTreg" group, $n = 5$). Additional experimental groups were control (no Treg, $n = 9$) and mice receiving only 1 Treg dose at day 0 ("1xTreg"). All animals consecutively received 4 weekly IV injections of BDD-FVIII for 1 month. (**e**) Inhibitor titers (BU/ml) as a function of time. (**f**) Anti-FVIII IgG titers (ng/ml) as a function of time. Data are average ±SD. Statistically significant differences are indicated for each time point.

Upon transferring polyclonal Tregs recipient mice develop antigen-specific suppression

The fact that we observed suppression of inhibitor formation well beyond when transplanted Treg levels had declined to undetectable could be explained by an escape from detection. Alternatively, we hypothesized that an efficient FVIII-specific suppressive response had been generated. To address this point, secondary transfer of Treg was attempted. CD4$^+$CD25$^+$ splenocytes from hemophilia A mice that had previously received *ex vivo* expanded Treg followed by 1 month of FVIII therapy were able to significantly suppress inhibitor/antibody formation against BDD-FVIII upon transfer to naive mice of the same strain (Figure 6a). Additionally, we failed to transfer suppression against anti-FVIII formation when CD4$^+$CD25$^+$ splenocytes were isolated from hemophilia A mice that had been treated with FVIII without having received the expanded Treg (Figure 6b). Importantly, no suppression of antibody formation against a different antigen (human FIX) was observed, indicating induction of antigen-specific Treg (Figure 6c). In summary, the data suggest that Treg therapy, using expanded nonspecific polyclonal Treg, followed by FVIII antigen administration ultimately induces a FVIII-specific Treg response.

Studies in which Treg cells were infused into human subjects indicated that Treg can have a long-term effect despite being rapidly deleted from the periphery by day 14, implying that transplanted Treg aid in the induction of endogenous Treg.[8] Similarly, the transfer of freshly isolated Treg also suggested that transplanted Treg promote induction of endogenous Treg.[23,24] To provide more direct evidence for this concept, *ex vivo* expanded GFP$^+$ Treg were transferred into DO11.10-tg Rag-2$^{-/-}$ BALB/c mice. This mouse is transgenic for the ovalbumin (OVA)-specific T cell receptor DO11.10, but deficient in endogenous mature B or T cells and, importantly, lacks Treg (Figure 7a).[6,16] Subsequently, recipient mice were injected 3×/week for 3 weeks with OVA antigen (100 µg of OVA peptide, OVA amino acids 323–339, representing the DO11.10 T cell receptor-specific epitope presented by I-A^d). Mice receiving only Treg (but no OVA) and mice receiving only OVA (but no Treg) served as controls. At the end of this period, donor (CD4$^+$CD25$^+$FoxP3$^+$KJ1-26$^-$) and recipient (CD4$^+$CD25$^+$FoxP3$^+$KJ1-26$^+$) Treg in spleens of treated mice were quantified (OVA-specific T cells stain positive with the clonotypic KJ1-26 antibody). Consistent with prior experiments, administration of OVA antigen by itself was ineffective for Treg induction (Figure 7b).[16] Transplanted GFP$^+$ Treg were detected in the spleens of recipients at 4–8% of total CD4$^+$ T cells at the end of the experiment (data not shown), but without the addition of antigen also failed to induce endogenous Treg (Figure 7b). However, transplanted Treg combined with subsequent OVA administration induced the generation of endogenous KJ1-26$^+$ OVA-specific Treg to >1% of CD4$^+$ T cells (Figure 7b). KJ1-26$^+$ Treg were GFP- and, conversely, no KJ1-26$^+$ cells were detected among the transplanted GFP$^+$ Treg (Figure 7c). These data confirm that the observed OVA-specific Treg were induced by conversion of endogenous CD4$^+$ T cells rather than being derived from transplanted Treg. Longer persistence of transplanted Treg in the DO11.10-tg Rag-2$^{-/-}$ (compared to immune competent mice of the same strain background)

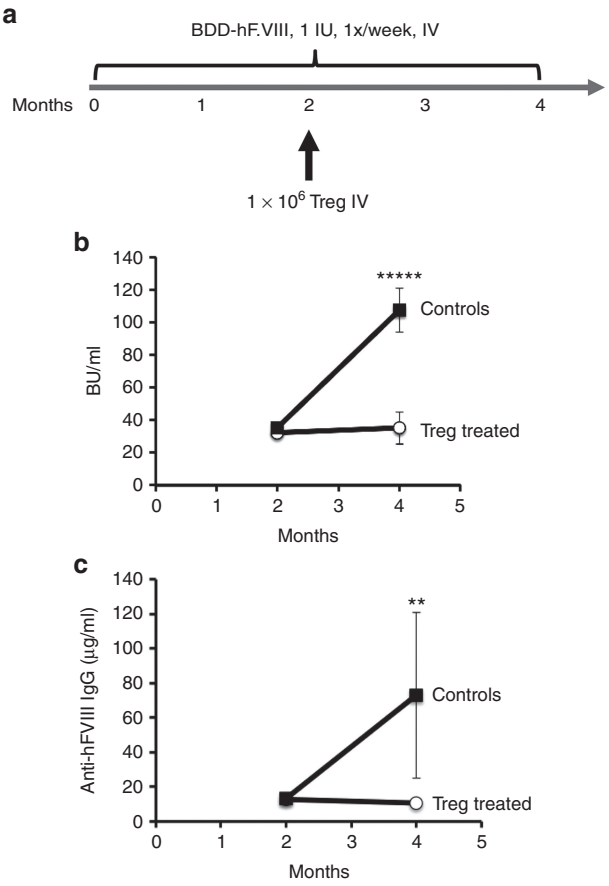


Figure 5 Expanded Treg suppress inhibitor formation against FVIII in hemophilia A mice with pre-existing response. (**a**) Experimental timeline. Hemophilia A mice (BALB/c *F8*e16$^{-/-}$) received weekly IV injections recombinant human B domain-deleted FVIII (BDD-FVIII, 1 IU per mouse and dose) for 2 months, resulting in inhibitor formation. A cohort of animals with similar inhibitor titers was selected and divided into two experimental groups ($n = 6$ each). One group received an IV injection of 1×10^6 expanded Treg ("Treg treated"; whereas "control" group did not receive cells). Weekly BDD-FVIII injections were continued in all animals for 2 more months. (**b**) Inhibitor titers (BU/ml) as a function of time. (**c**) Anti-FVIII IgG titers (ng/ml) as a function of time. Data are average ±SD. Statistically significant differences are indicated for each time point.

may be due to the lack of endogenous Treg in these mice, so that transplanted cells are able to occupy and persist in Treg niches.

Finally, we wanted to examine whether modification of DC expanded Treg may aid in ova-specific Treg induction. Consistent with data by Lan *et al.*, Treg primed bone marrow derived DC and splenic DC were able to induce the conversion of DO11.10$^+$ (KJ1-26$^+$) CD4$^+$CD25$^-$ Teff cells to DO11.10$^+$ (KJ1-26$^+$) CD4$^+$CD25$^+$FoxP3$^+$ Treg *in vitro,* following coculture of DC with DO11.10$^+$ Teff in the presence of OVA peptide for 3 days (Figure 7d,e).[26] Treg primed DC pulsed with OVA antigen induced KJ1-26$^+$ OVA-specific Treg to >1.5% of CD4$^+$ T cells, while Teff primed DC were comparatively ineffective in the induction of KJ1-26$^+$ OVA-specific Treg (Figure 7e). Nearly identical results were obtained in a second independent experiment (data not shown).

Ex vivo expanded polyclonal Treg suppress inhibitor formation in FIX gene replacement therapy without affecting the antibody response directed against vector capsid

Next, we assessed the utility of Treg therapy to prevent antibody formation in gene therapy. Muscle-directed gene transfer with an AAV1 vector expressing human FIX was used as the experimental model. The AAV1-CMV-FIX vector was injected intramuscularly (IM) into 8 hemophilia B mice with targeted *F9* gene deletion. Half of the animals had received 1×10^6 expanded Treg 2 days prior to gene transfer (Figure 8a). In this case, hemophilia B mice with coexpression of a GFP reporter and the transcription factor FoxP3 (generated by crossing FoxP3-IRES-GFP$^+$ BALB/c x F9$^{-/-}$ BALB/c) were the source of GFP$^+$ Treg for *ex vivo* expansion. Control mice (no Treg treatment) developed high-titer inhibitors against FIX (on average 8–10 BU/ml) for the duration of the experiment (2.5 months). In contrast, Treg therapy prevented inhibitor formation and substantially suppressed the total IgG response to FIX (Figure 8b,c). Treg treated mice only transiently formed lower titer anti-FIX, while control mice had significantly higher titers, which declined somewhat over time albeit with only little change in Bethesda titer (Figure 8b,c). Systemic FIX antigen levels were significantly higher for Treg treated mice 6 weeks after gene transfer, while levels caught up in control mice by 10 weeks, suggesting that the inhibitors failed to clear FIX at this time point in the control animals (Figure 8d). Coagulation times were consistently shorter in Treg treated mice for the duration of the experiments (Figure 8e). Introduction of Treg cells had, however, no effect on antibody formation against the viral capsid (including neutralizing titers). (Figure 8f,g). When Treg-treated mice were challenged at a later time point with FIX in adjuvant, three of four animals remained unresponsive to FIX (data not shown). Taken together, the outcomes of these experiments indicate that in contrast to expanded polyclonal Treg, induced antigen-specific Treg do not display a strong *in vivo* "bystander effect."

DISCUSSION

The therapeutic potential of CD4$^+$CD25$^+$FoxP3$^+$ Treg in the improvement of autoimmunity and transplant tolerance has been well documented (http://clinicaltrials.gov/ NCT01624077, NCT01210664, NCT00602693).[8–11,27] Our new data demonstrate that the same Treg technology currently used in clinical trials for autoimmune and GvHD can be applied to reduce the risk and strength of immune responses in treatment of inherited protein deficiencies such as hemophilia. This relatively simple concept is an attractive adjunct therapy to current treatment modalities. There is no requirement to identify specific or immunodominant epitopes or for gene manipulation of the cells when using endogenous polyclonal Treg. Utilizing the body's own means of immune regulation is a natural and comparatively safe way to achieve suppression, while avoiding strong general immunosuppression or other potentially severe side effects of small molecule or antibody drugs.

Evolving technology of *ex vivo* Treg expansion facilitates preclinical and translational studies

Ex vivo expansion of Treg provides a means of generating sufficient cell doses while starting with limited numbers of autologous Treg recovered from a patient. Any such protocol needs to balance the need for cell expansion with the requirement for maintaining a suppressor phenotype. Initial contamination with effector T cells (Teff) may eventually result in outgrowth of these cells. Repeated restimulations of Treg, while causing an increase in yield of expanded Treg, can also lead to loss of FoxP3 expression and corresponding loss in functionality.[28,29] Attempts to conserve FoxP3 expression include the addition of rapamycin, all trans retinoic acid, and also the addition of cytokines like IL-10 or transforming growth factor-β that can cause CD4$^+$ Teff to become induced Treg.[30–33] Inhibition of mTOR with rapamycin results in deletion of Teff while Treg continue to expand.[2,3] Although

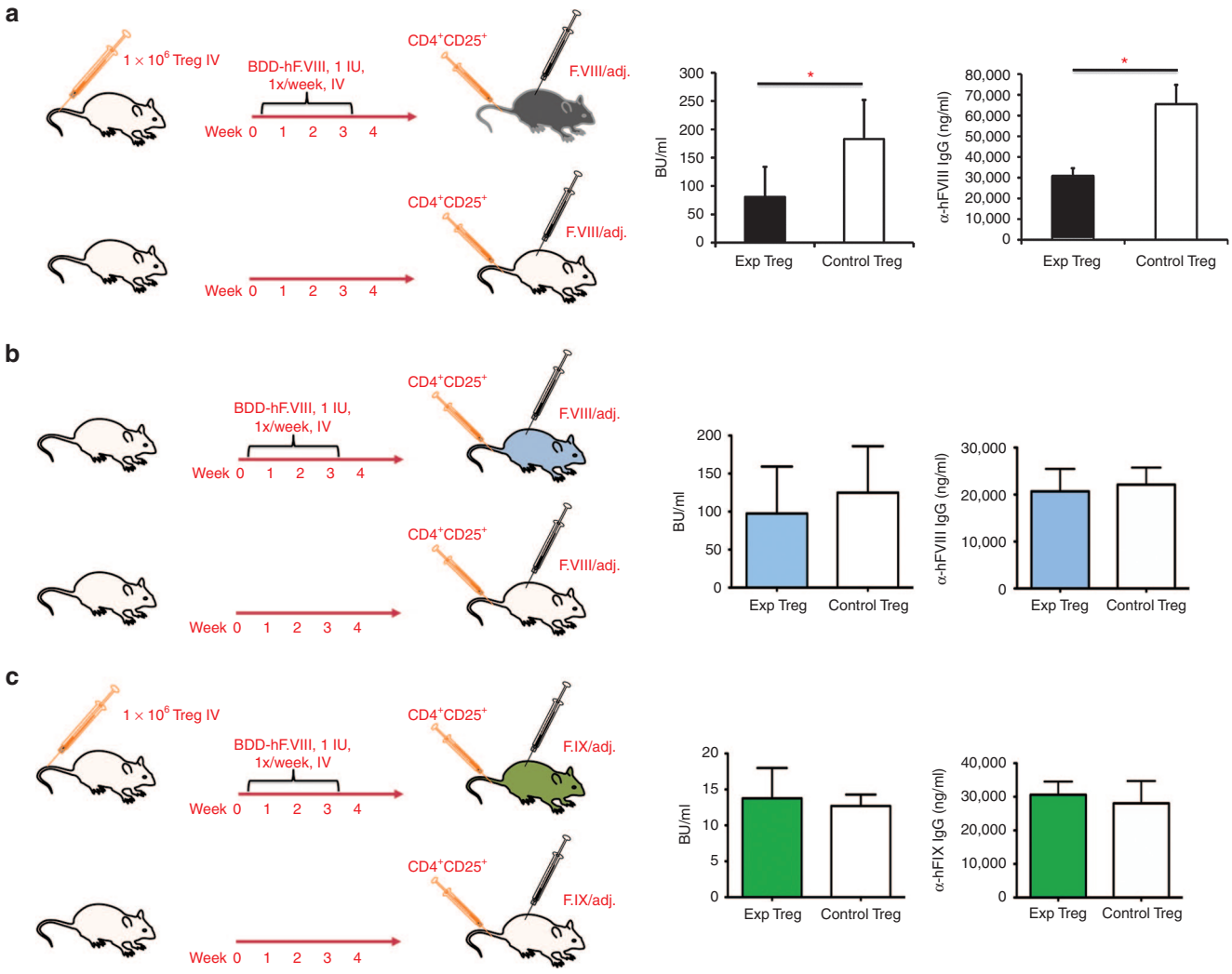


Figure 6 Secondary transfer of CD4+CD25+ Treg from BDD-FVIII treated hemophilia A mice. Naive hemophilia A mice (BALB/c *F8*e16$^{-/-}$) received IV injections of 1×10^6 freshly isolated and magnetically purified splenic CD4+CD25+ T cells. (**a**) Adoptive transfer from mice that had received Treg therapy (1×10^6 *ex vivo* expanded Treg) followed by 1 month of weekly BDD-FVIII injections (1 IU/dose, IV). Recipient mice were immunized with BDD-FVIII. Resulting inhibitor titers (BU/ml) and anti-FVIII IgG titers (ng/ml) were shown in back bars. In this and all other panels, results were compared to antibody titers in mice that were immunized after receiving CD4+CD25+ T cells from naive mice (white bars). (**b**) Adoptive transfer from mice that had received 1 month of weekly BDD-FVIII injections (but no Treg). Recipient mice were immunized with BDD-FVIII. Resulting inhibitor titers (BU/ml) and anti-FVIII IgG titers (ng/ml) were shown in blue bars. (**c**) Adoptive transfer from mice that had received Treg therapy (1×10^6 *ex vivo* expanded Treg) followed by 1 month of weekly BDD-FVIII injections (1 IU/dose, IV). Recipient mice were immunized with FIX. Resulting inhibitor titers (BU/ml) and anti-FIX IgG titers (ng/ml) were shown in green bars.

used in some clinical Treg expansion protocols, this method slows expansion of the Treg culture and thus can lead to a significant loss in yield.[31] Because of these difficulties, relatively few studies on therapy with *ex vivo* expanded Treg in murine models have been published. We solved these hurdles by starting with highly purified Treg, isolated from FoxP3-GFP reporter mice, and optimizing expansion and time of culture. In the combined experience between our laboratories, we noticed that the batch of serum might influence the extent of Treg expansion, while the source and batch of antibody-coated beads can affect the ability to expand cells that maintain FoxP3 expression. Therefore, a careful characterization of a test expansion culture should be carried out when using novel reagents.

Translation of the approach is greatly facilitated by the fact that the methodology for isolation and expansion of human Treg has been refined and is actually superior to that available for murine Treg. Human Treg are typically purified by multi-step magnetic or flow sorting to ultimately obtain CD4+CD25hiCD127lo cells. Moreover, artificial antigen presenting cell (aAPC) lines have been established that provide superior results to beads, leading to massive expansion of functional Treg.[32] These aAPCs express a costimulatory molecule such as CD86 or 41-BB and overexpress a high-affinity Fc receptor, so that they can be coated with anti-CD3.[34] GMP guidelines have been defined for the manufacture of Treg for clinical use.[8,35,36] We have recently shown that soluble Fc-GITR-Ligand, combined with anti-CD3 beads, robustly expands Treg *in vitro*, representing a potentially alternative technology.[37]

Polyclonal versus antigen-specific Treg

Somewhat to our surprise, polyclonal Treg were consistently effective in suppressing inhibitor formation in different treatment scenarios in gene and protein replacement therapy for hemophilia. For the experiments presented here, we chose a Treg dose similar to the currently highest doses used in clinical trials for T1D and Crohn's disease (for a 20–25 g mouse, a total dose of 1×10^6 cells represents a

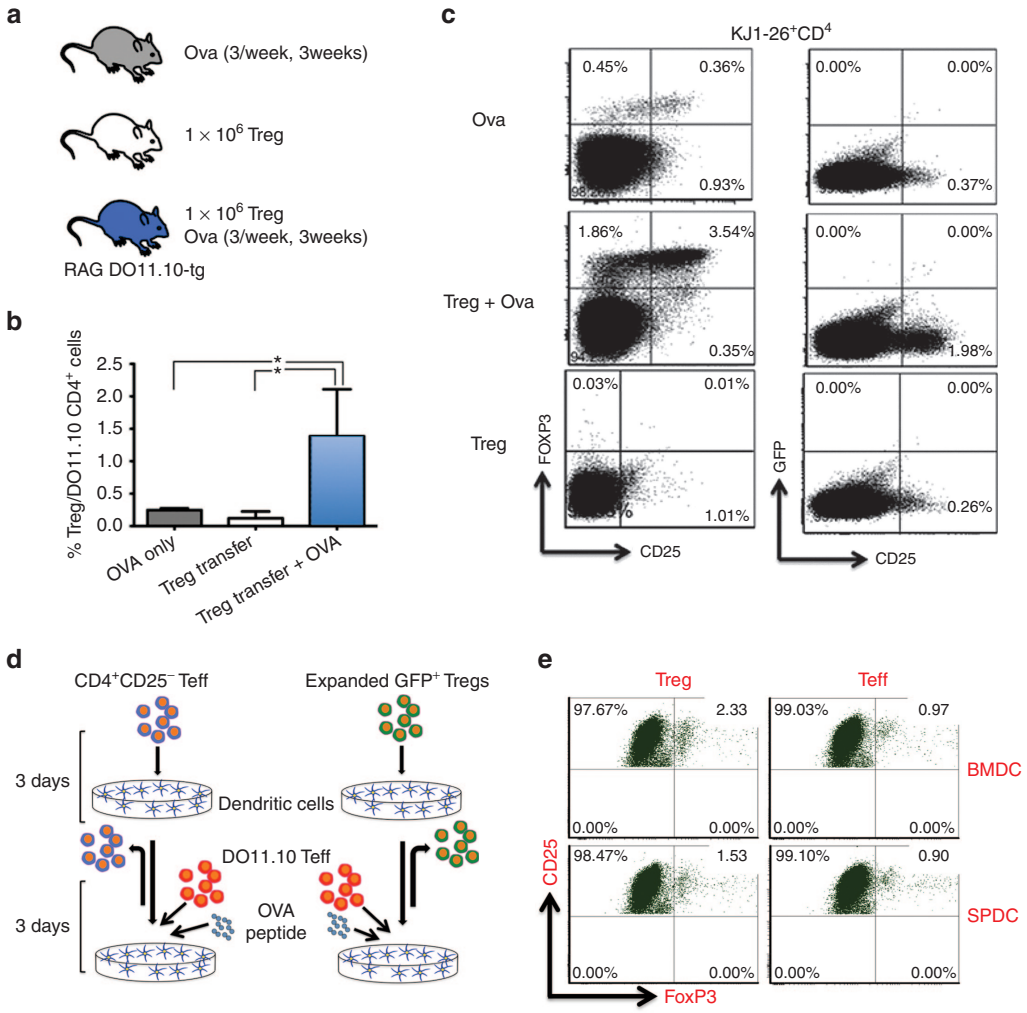


Figure 7 Induction of ovalbumin (OVA)-specific Treg in DO11.10-tg *Rag-2*$^{-/-}$ BALB/c mice (which are transgenic for OVA-specific CD4$^+$ TCR and lack endogenous Treg). (**a**) Experimental groups: Group I (grey): mice were injected IP 3-times per week with OVA peptide antigen. Group II (white): mice received a single dose of *ex vivo* expanded polyclonal Treg (1×10^6 cells/mouse, IV) at day 0. Group III (blue): mice received a single dose of *ex vivo* expanded Treg (1×10^6 cells/mouse, IV) followed by IP injections, three times per week with OVA peptide antigen. (**b**) Frequency of endogenous OVA-specific CD4$^+$CD25$^+$FoxP3$^+$ Treg (1 month after initiation of experiment; $n = 6$/group). (**c**) Flow cytometric analysis demonstrating that no OVA-specific (K1J1-26$^+$) Treg were detected among injected Treg, which are identified by their GFP expression (no cell permeabilization was applied for these stains shown in right panels, GFP versus CD25, to preserve maximum GFP signal). (**d**) Bone marrow derived DC (BMDC) and splenic DC (SPDC) primed with either Teff or expanded Treg were isolated and cocultured with CD4$^+$CD25$^-$ Teff cells from DO11.10 *Rag-2*$^{-/-}$ mice in the presence of 10 µg/ml OVA peptide. (**e**) Induction of OVA specific DO11.10$^+$ CD4$^+$CD25$^+$FoxP3$^+$ Treg was determined by flow cytometry.

dose of 4–5 × 10^7 cells/kg). Antigen-specific Treg are known to be effective at lower cell doses and thus may be preferable for tolerance induction. However, the frequency of antigen-specific Treg is expected to be less than 0.1% of CD4$^+$ T cell, making development of a protocol more challenging and complex.[38,39] It may not be possible to isolate antigen-specific Treg from individuals with no or limited exposure to the therapeutic protein. Therefore, the approach of using of polyclonal Treg is likely more universally applicable.

At the same time, there are limitations to the use of polyclonal Treg. One concern is nonspecific immune suppression early after Treg transplant. However, when a protein with multiple strong B cell epitopes (keyhole limpet hemocyanin, KLH) was coadministered with FVIII, statistically significant suppression of antibody formation was observed for FVIII but not for KLH (data not shown). Similarly, the lack of suppression of the NAB response to viral particles (which display multiple repetitive epitopes) in the AAV gene transfer experiment suggests that Treg therapy does not suppress the immune system to the extent that it cannot mount a proper response to pathogens such as viruses. On the other hand, Treg are not suitable to obtain the ability to readminister vector, and they may not be sufficiently effective if strong immune suppression is required, which may be the case for some gene therapies.[40] In a scenario where stronger Treg-mediated suppression may be required to promote robust immune tolerance to a therapeutic protein, generation of antigen-specific Treg may be necessary. For example, we have previously shown that a 1-month course of repeated coadministration of peptide (representing CD4$^+$ T cells epitopes) or protein antigens with rapamycin induces antigen-specific Treg and depletes Teff *in vivo*.[16,41,42] These could then be expanded *in vitro* upon restimulation with antigen.[41] Rapamycin may also further enhance the potency of polyclonal Treg therapy.

Mechanism of suppression by polyclonal Treg, further optimization, and potential clinical applications

Our study, in addition to reports of others, suggest a mechanism in which administration of polyclonal Treg establishes an immune

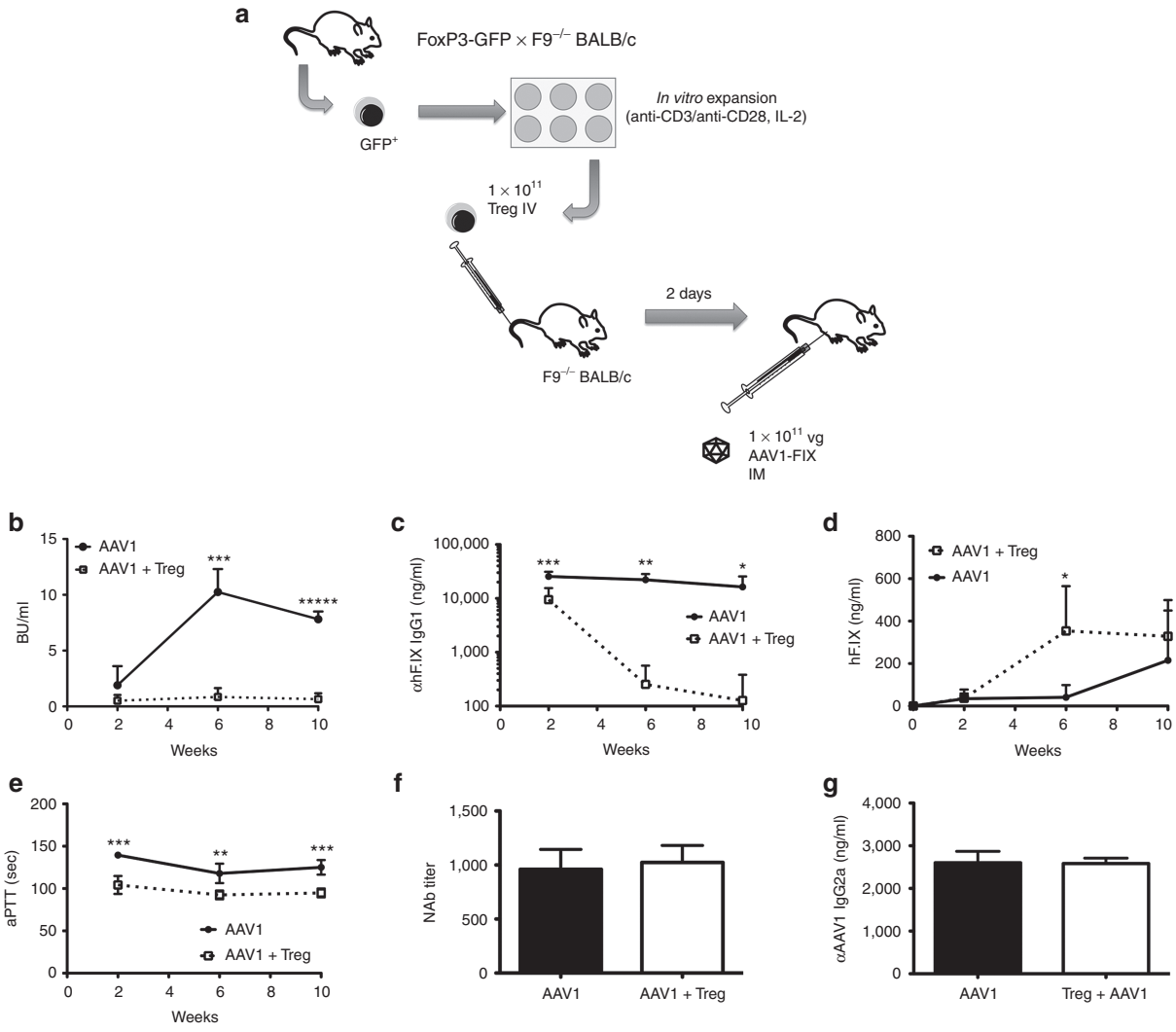


Figure 8 Suppression of inhibitor formation against FIX by *ex vivo* expanded $CD4^+CD25^+FoxP3^+$ Treg that had been isolated from hemophilia B mice with a GFP reporter gene knock-in at FoxP3 locus (generated by crossing BALB/c *F9*$^{-/-}$ x FoxP3-GFP mice). (**a**) Experimental outline. *Ex vivo* expanded Treg (1×10^6) were injected IV into hemophilia B mice (BALB/c *F9*$^{-/-}$) followed by muscle-directed gene transfer with AAV1-CMV-FIX vector ("AAV1+Treg" group"). Control mice ("AAV1" group) received vector but no Treg. The vector dose was 1 × 1011 vg/mouse for all animals ($n = 4$ per experimental group). (**b**) Inhibitor titers (BU/ml) as a function of time after vector administration. (**c**) Anti-FIX IgG titers (ng/ml). (**d**) Systemic FIX antigen titers (ng/ml). (**e**) Coagulation times (activated partial thromboplastin times, aPTT, in seconds) of plasma samples. (**f**) Neutralizing antibody titers (reciprocal) against AAV1 vector 1 month after vector administration. (**g**) IgG2a anti-capsid titers (ng/ml) 1 month after vector administration. Data are average ±SD with statistically significant differences between experimental groups indicated.

suppressive environment that limits responses to the therapeutic protein during initial exposure to the antigen, possibly due to bystander suppression as a result of the reduction in costimulation.[23,24,38] *Ex vivo* expanded Treg are functionally superior to freshly isolated Treg.[29] For example, we found that expanded Treg populated various compartments at higher frequencies. Furthermore, *ex vivo* expansion caused a marked upregulation of CTLA-4. Others have shown that Treg trans-endocytose CD80/86 costimulatory molecules upon binding to CTLA-4.[43] Therefore, upregulation of CTLA-4 is likely responsible for the substantial reduction of CD80/86 on DC that we observed after coculture with expanded Treg. Over time, antigen-specific suppression emerges, entirely or at least in part because of induction of endogenous Treg, which is facilitated by the transplanted Treg. Previously, we found evidence that FVIII injections into hemophilia A mice on BALB/c background activated both Teff and Treg (which failed to suppress inhibitor formation).[44] Transplant of *ex vivo* expanded Treg, however, tilts the balance toward the Treg response, which is now capable of specifically suppressing antibody formation against FVIII. *Ex vivo* expanded Treg persist only transiently after transplant. Therefore, the time interval during which the therapeutic antigen and injected Treg are present at the same time is limited in our FVIII protein therapy experiments. In order to achieve long-term tolerance in this model, FVIII antigen administration and repeat Treg dosing needs to be optimized to accomplish optimal induction of FVIII-specific Treg.

Several potential applications in treatment of hemophilia or other genetic diseases present themselves. Treg therapy at the time of initial exposure to the coagulation factor or other therapeutic proteins (such as in enzyme replacement therapy for lysosomal storage disorders) could further reduce the risk of an adaptive immune response. Treg administration may serve as an adjunct therapy to current immune tolerance induction protocols in hemophilia and accelerate the reversal of inhibitor formation. Treg therapy at the time of vector administration could reduce the risk of unwanted

immune responses in gene therapy, especially for more immunogenic routes of vector administration/target organs and transgenes. Recent studies have suggested that in humans Treg may counteract inflammatory CD8$^+$ T cell responses against AAV capsid antigen in skeletal muscle, thereby helping to resolve inflammation and preserving transgene expression.[45,46] It is conceivable that this effect could be further enhanced by an adjunct therapy using *ex vivo* expanded autologous Treg.

MATERIALS AND METHODS

Mice

All animals used at the onset of the experiments were 8–10 week old male mice of the BALB/c background. BALB/c wt and CByJ.PL(B6)-*Thy1a*/ScrJ (thy1.1) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Hemophilia A mice with a deletion in exon 16 of the F8 gene (BALB/c *F8*e16$^{-/-}$) were provided by Dr. David Lillicrap (Queens, Ontario, Canada). Hemophilia B mice with a targeted deletion of the promoter and the first 3 exons of the *F9* gene had been backcrossed onto a BALB/c background for >10 generations.[47] DO11.10-tg Rag2$^{-/-}$ mice with a T cell receptor specific for a 323–339 of chicken OVA, presented by MHCII I-Ad, were originally obtained from Taconic (Hudson, NY). BALB/c mice with reporter gene knocked-in at FoxP3 locus (FoxP3-IRES-eGFP) were as previously described and kindly provided by Dr. Talal Chatila.[48] Hemophilia B mice with GFP expression in FoxP3$^+$ cells were generated by crossing BALB/c F9$^{-/-}$ mice with FoxP3-IRES-eGFP BALB/c mice. Approximately 1 of 10 offspring showed the crossover event necessary to generate the F9 knock-out/FoxP3-GFP knock-in genotype (both genes are located on the X chromosome).[7] Subsequent breeding generated homozygous females and hemizygous males. Animals were housed under special pathogen-free conditions at the University of Florida and treated under Institutional Animal Care and Use Committee-approved protocols.

Viral vectors

The single-stranded AAV serotype 1 vector expressing human *F9* cDNA under the control of the cytomegalovirus immediate early enhance/promoter (AAV1-CMV-FIX) were as described.[49,50] Vectors were produced by triple transfection of HEK-293 cells, purified by differential precipitation with polyethylene glycol followed by CsCl gradient centrifugation, filter sterilized, and quantified by silver staining and slot-blot hybridization as described elsewhere.[51] Adenovirus serotype 5 expressing enhanced GFP under the CMV promoter (AD-CMV-GFP) was purchased from Vector Biolabs (Philadelphia, PA).

Antibodies and reagents

Purified anti-CD16/32 (Fc Block), anti-CD4 (V500), anti-CD62L (PerCP-Cy5.5), anti-CD86 (V450) antibodies were purchased from BD Biosciences (San Jose, CA); anti-CD152 (BV421), anti-CD25 (BV605), anti-helios (PE) antibodies were purchased from Biolegend (San Diego, CA); anti-CD4 (eFluor 450), anti-thy-1.1 (PerCP-Cy5.5), anti-thy1.1 (APC), anti-thy1.2 (PE), FoxP3 (Alexa Fluor 647), anti-KJ1-26 (PE), anti-CD80 (PE) antibodies were from eBioscience (San Diego, CA). Pro5 recombinant murine MHC class I pentamers (PE) to enumerate GFP specific CD8$^+$ T lymphocytes were from Proimmune (Oxford, UK). Mouse CD4$^+$ T cell isolation kit, mouse pan DC isolation kit, LD, LS and MS columns, MACSiMag separator and mouse Treg expansion kit (CD3/CD28 paramagnetic beads) were from Miltenyi Biotec (Auburn, CA). Mouse T-activator CD3/CD28 paramagnetic Dynabeads and CellTrace Violet cell proliferation kit were from Life Technologies (Grand Island, NY). OVA peptide (323–339) was synthesized by Anaspec (Fremont, CA). Rapamycin was from LC laboratories (Woburn, MA). Recombinant BDD-FVIII (Xyntha) was from Wyeth Pharmaceuticals (Philadelphia, PA). FVIII deficient plasma and FIX deficient plasma were from Haematologic Technologies (Essex Junction, VT).

Ex vivo expansion of Treg

Spleens of BALB/c FoxP3-IRES-eGFP mice were homogenized and enriched for CD4$^+$ T cells by magnetic depletion of nontarget cells over an LS column (Miltenyi Biotec). GFP$^+$ cells, representing the Treg population of CD4$^+$ T cells (~10%), were isolated using the FACSAria II cell sorter (BD Biosciences). Treg populations were analyzed for purity post sort to assure purity (≥95 GFP$^+$). Treg were cultured in RPMI-1640 media (Life Technologies) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, 1X GlutaMAX-1, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 1X nonessential amino acids and 10 µmol/l 2-mercaptoethanol (Life Technologies). Recombinant hIL-2 (Proleukin/aldesleukin, Prometheus Therapeutics and Diagnostics, San Diego, CA) was added at a concentration of 2,000 IU/ml. Cells (1×10^6 Treg/ml) were seeded into 12-well plates coated with CD3/CD28 microbeads (Miltenyi or Dynabeads) at a 2:1 ratio of cells to beads. Culture volume was doubled by adding fresh medium and hIL-2 on days 2, 4, 6, 9, and 11 assuming consumption, in order to maintain a high hIL-2 concentration. Cells were monitored for an increase in cell size and change in shape and restimulated on approximately day 7 by removing CD3/CD28 beads on a magnetic separator. Cells were restimulated with fresh beads at a 1:1 cell to bead ratio. Cells were cultured for 11–14 days. Purity of the GFP$^+$ population and FoxP3 expression was closely monitored at regular intervals by flow cytometry during expansion.

Biodistribution of transplanted Treg

Immunocompetent thy1.1 (CD90.1) mice were injected with 1×10^6 *ex vivo* expanded thy1.2$^+$ (CD90.2) Treg or freshly isolated Treg by tail-vein injection. The persistence of GFP$^+$thy1.2$^+$FoxP3$^+$ Treg was tracked in whole blood, spleens and lymph nodes of transfer recipients on days 1, 2, 7, 14, and 21 after transfer. CD8$^+$ T cell responses to GFP were enumerated by injecting mice with 1×10^6 *ex vivo* expanded Treg and blood was analyzed 10 days later with the MHC class I pentamer to GFP. As a positive control for CD8$^+$ T cell responses to GFP, mice were injected with 5×108 plaque forming units of AD5-CMV-GFP by the intramuscular route.

Phenotypic characterization of expanded populations

Freshly isolated or expanded Treg were evaluated for expression of CD4, CD25, GFP, and FoxP3. Intracellular staining for FoxP3 was performed with the FoxP3 staining kit (eBioscience). Surface labeling of GFP$^+$ cells and intracellular staining for FoxP3 was carried out in parallel for all experiments. Cells were stained for Treg associated markers CTLA-4 (CD152), CD62L, and helios. Briefly, 1×10^5–1×10^6 cells in a volume of 100 µl were blocked with CD16/32 for 15 minutes and surface labeled with antibodies at recommended concentrations. Fixation-permeabilization carried out as required and intracellular antibodies were added. Samples were acquired on the LSR II flow cytometer (BD Biosciences) and analyzed using FCS Express 4 (DeNovo Software, Los Angeles, CA). The induction of tolerogenic DC by *ex vivo* expanded Treg was examined by coculturing splenic and bone marrow isolated pan DC with either freshly isolated CD4$^+$CD25$^+$ Treg cells, *ex vivo* expanded Treg or CD4$^+$CD25$^-$ effector T cells (Teff). A 5:1 ratio of Treg or Teff cells to DC were cocultured for 3 days in the presence of recombinant murine GM-CSF (20 ng/ml), recombinant murine IL-4 (20 ng/ml), recombinant murine IL-3 (20 ng/ml) and CD3/CD28 microbeads, at the end of which CD80 and CD86 costimulatory molecule expression was analyzed by flow cytometry. Treg or Teff primed DC were isolated by removal of CD4$^+$ T cells and cultured with freshly isolated CD4$^+$CD25$^-$ Teff from DO11.10-tg Rag2$^{-/-}$ mice, which lack Treg and are OVA peptide specific. Cells were cultured for another 3 days in the presence of 100 IU/ml hIL-2 and 10 µg/ml OVA peptide. The induction of DO11.10$^+$ CD4$^+$CD25$^+$FoxP3$^+$Treg by tolerogenic DC was analyzed by flow cytometry.

In vitro suppression assay

In vitro suppression by expanded Treg was based on their capacity to suppress the proliferation of allogeneic CD4$^+$ responder T cells. Spleens from BALB/c wt mice were homogenized and RBC lysed using the 1X RBC lysis buffer (eBioscience). Splenocytes were labeled with 3 µmol/l CellTrace Violet for 20 minutes at 37 °C. GFP$^+$ *ex vivo* expanded Treg (2×10^5) and CellTrace Violet labeled responder splenocytes were seeded at effector:responder ratios of 1:1, 1:4, 1:16, 1:32 and stimulated with CD3/CD28 magnetic beads at a 1:1 ratio of cells to beads for 72 hours at 37 °C. Cells were resuspended and stained with anti-CD4 APC antibody to assess proliferation of responder CD4$^+$ T cells. GFP was used to discriminate between responder cells and Treg. Proliferation was determined by quantitating CellTrace Violet fluorescence intensity relative to a parent population of unstimulated responder cells (0% proliferation) and stimulated cells incubated without Treg (100% proliferation). Percentage of CD4$^+$ responder T cell proliferation was also determined using ModFit LT analysis on FCS Express 4.

Protein and gene therapy experiments

Hemophilia A mice received 1IU of BDD-FVIII in 200 µl of phosphate-buffered saline by weekly tail-vein injections. In one experiment, KLH (Sigma-Aldrich, St Louis, MO) was coadministered with FVIII at a dose of 100 µg. Hemophilia

B mice received AAV1-CMV-FIX at 1×10^{11} vector genomes (vg)/mouse. Viral vector was administered intramuscularly into the quadriceps and tibialis anterior of one hind limb as previously described.[47] Plasma samples were collected by tail bleed into 0.38% sodium citrate buffer as described.[47]

Analysis of plasma samples

Plasma samples were analyzed using a modified activated partial thromboplastin time assay (aPTT). Inhibitory antibodies to FVIII or FIX were measured by Bethesda assay as described.[47] One Bethesda unit is defined as the reciprocal of the dilution of test plasma at which 50% of FVIII or FIX activity is inhibited. Measurements were carried out in a Diagnostica Stago STart Hemostasis Analyzer (Parsippany, NJ). Enzyme-linked immunosorbent assay (ELISA)-based measurements of FIX antigen and IgG antibodies to FVIII and FIX in murine plasma samples were carried out as described.[47] Capsid specific IgG2a antibodies against AAV1 were detected by ELISA as published.[49] Sample wells were coated with 2.5×10^9 vg/well intact AAV1 particles. Neutralizing antibodies to AAV1 capsid were titrated by incubating serially diluted plasma samples with 5×10^9 vg/well of self-complementary AAV1-CMV-GFP in a U-bottom 96-well low-adherence plate for 1 hour at 37 °C. The virus-plasma mix was then overlayed on human adenovirus 5 (Ad5) infected HEK-293 cells and incubated for 48 hours at 37 °C. Neutralizing antibody (Nab) titers are expressed as the reciprocal of the plasma dilution required to neutralize 50% of GFP expression compared to controls in the absence of test plasma.

Treg transfer studies and adoptive transfer of suppression

Transfer of *ex vivo* expanded Treg into hemophilia A and B mice were generally performed by tail-vein injection of 1×10^6 cells. Mice were either injected with a single dose of Treg or repeatedly injected with three doses of Treg spaced 1–2 weeks apart. Mice continued to receive 1 IU of BDD-FVIII weekly throughout the course of Treg injections. Secondary transfer experiments from mice that had previously received *ex vivo* expanded Treg or control animals were carried out by extraction of splenic $CD4^+CD25^+$ cells. These were pooled from 5–6 mice for each experimental group and adoptively transferred to naive strain-matched mice by tail-vein injection of 1×10^6 cells/mouse as described previously.[15] Recipient mice (4–6 mice/experimental group) were immunized the next day by subcutaneous administration of 1 IU of BDD-FVIII or FIX in Sigma Adjuvant System (Sigma-Aldrich). In case of BDD-FVIII, a booster immunization was performed 2 weeks later. Antibody titers against BDD-FVIII or FIX were measured 1 month after adoptive Treg transfer.

Statistical analysis

Statistical significance was determined with an unpaired, two-tailed Student's *t*-test using GraphPad Prism 5 software (La Jolla, CA). Values at $P < 0.05$ were deemed significant and indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

CONFLICT OF INTEREST

R.W.H. has been receiving royalty payments from Genzyme for license of AAV-FIX technology. All other authors declare no conflict of interest.

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