Role of antigen-specific regulatory CD4$^+$ CD25$^+$ T cells in tolerance induction after neonatal IP administration of AAV-hF.IX

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Neonatal AAV8-mediated Factor IX (F.IX) gene delivery was applied as a model for exploring mechanisms of tolerance induction during immune ontogeny. Intraperitoneal delivery of AAV8/ Factor IX (hF.IX) during weeks 1–4 of life, over a 20-fold dose range, directed stable hF.IX expression, correction of coagulopathy in F.IX-null hemophilia B mice, and induction of tolerance to hF.IX; however, only primary injection at 1–2 days of life enabled increasing AAV8-mediated hF.IX expression after re-administration, due to the absence of anti-viral capsid antibodies. Adoptive splenocyte transfer from tolerized mice demonstrated induction of CD4$^+$ CD25$^+$ T regulatory (T$_{reg}$) populations that specifically suppressed anti-hF.IX antibody responses, but not responses to third party antigen. Induction of hF.IX antibodies was only observed in tolerized mice after in vivo CD4$^+$ CD25$^+$ T cell depletion and hF.IX challenge. Thus, primary injection of AAV during a critical period in the first week of life does not elicit antiviral responses, enabling re-administration of AAV and augmentation of hF.IX levels. Expansion of hF.IX-specific CD4$^+$ CD25$^+$ T$_{reg}$ has a major role in tolerance induction early in immune ontogeny. Neonatal gene transfer provides a useful approach for defining the ontogeny of immune responses and may suggest approaches for inducing tolerance in the context of genetic therapies.

Introduction

There have been significant advances in approaches for genetic correction of hemophilia B. However, in many studies, adaptive and innate immune responses stimulated by viral gene transfer vectors mediating Factor IX (F.IX) expression have limited effective correction of this coagulopathy. Recent advances in viral vector design, techniques for somatic gene modification, and approaches to address inhibitory immune responses are increasing the likelihood that genetic therapies will be soon be successful. Adeno-associated viral vectors (AAV) have been intensively investigated in pre-clinical and clinical Phase 1 studies of F.IX gene therapy. Identification of novel AAV serotypes enabled progress in improving transduction efficiency and decreasing immunogenicity. Thus, increased F.IX expression was achieved with delivery of AAV5-F.IX, and intravenous (IV) injection of AAV2-F.IX genomes cross-packaged into AAV-2/1, -2/4 and -2/6 vectors. AAV1-mediated F.IX delivery also improved correction of hF.IX levels in large animal models of hemophilia B. AAV7 and 8 were more efficient than AAV2 and AAV5-pseudotyped vectors for intramuscular (IM) gene delivery and viral promoters were more active than muscle-specific promoters. AAV8-F.IX vectors also showed superior hepatic transduction with intraportal delivery in mice and non-human primates. Despite the lack of pathogenicity of these vectors in preclinical studies, early clinical trials were halted due to the unexpected development of hepatitis and extinction of transgene expression due to antiviral capsid-related antigen immune responses in patients receiving higher doses of AAV2-hF.IX. Studies by Nayak et al. showed that immune responses and F.IX gene expression were both AAV serotype and mouse strain-restricted, suggesting that in the clinical setting, immune responses will be regulated at some level by human leukocyte antigen (HLA) sub-types in patient populations.

Lentiviral-mediated gene transfer has also been applied to genetic therapy of F.IX deficiency. Intra-portal delivery of lenti-F.IX vectors produced sustained F.IX expression in the absence of cell division, and high level F.IX expression was observed after transduction of hematopoietic stem cells with lentivectors expressing F.IX. To address concerns regarding insertional mutagenesis, hepatocyte-targeted integrase-deficient lentivectors (IDLV) have been tested and produced robust F.IX gene expression without induction of neutralizing antibody responses in hemophilic mice.

More recently, the role of innate inflammatory responses affecting lentiviral transduction and F.IX gene expression has been examined. Pre-treatment with dexamethasone, to suppress both toll-like receptor (TLR) and non-TLR innate responses, normalized expression of interferon-induced genes and increased hepatocyte transduction and F.IX levels after administration of lenti-F.IX. These studies support not only the promise of genetic therapies, but also point to the continuing challenges presented by host-mediated innate and adaptive immune responses to therapeutic transgene and vectors. In utero and neonatal models of gene delivery have been explored as potential strategies for (1) early correction of genetic diseases, (2) models for induction of tolerance early in immune ontogeny, and (3) as a sensitive measure of toxic and oncogenic off-target effects of viral gene therapy. In contrast to the immune responses associated with viral-mediated gene delivery in adults, in utero injection of either adenoviral (Ad) or AAV vectors does not
evoke immune responses in murine models. For example, re-administration in adults of Ad-F.IX or Ad-F.VIII, after in utero IP administration, produced similar coagulant levels as those achieved in naive adults. With further Ad-F.IX or Ad-F.VIII injections, anti-adenoviral immune responses abrogated gene expression indicating that the initial lack of responses was due to immune ignorance rather than induction of tolerance. 

Retroviral-mediated delivery of the canine FIX gene in newborn mice and dogs also directed therapeutic levels of FIX expression, but humoral responses to FIX were detected with subsequent protein injections. Injection of a variety of AAV serotypes in neonatal models does not elicit FIX antibody responses. The value of neonatal AAV-mediated gene delivery models has recently been underscored by the successful correction of neurologic, skeletal and metabolic abnormalities in a murine model of mucopolysaccharidosis (MPS I).

Studies with intra-hepatic AAV-F.IX delivery in adult transgenic models have defined the role of antigen-specific T regulatory cells (Tregs) in the successful induction of immune tolerance. In adults, Tregs represent 5–12% of CD4+ T cells and include both quiescent populations, and cells that continuously replicate in the presence of tissue self-antigens. An important distinction between mice, as compared with sheep, non-human primates and humans, is that mice are not immunologically ignorant rather than induction of tolerance. Dose escalation of AAV8-hFIX administration in neonates produces increasing levels of hFIX without induction of a humoral response.

RESULTS

IP delivery of AAV8-CBA-hFIX in BALB/c neonates achieves stable, high level expression of hFIX without induction of humoral response.

Strain-specific differences in the immune responses to gene delivery vectors and hFIX have been demonstrated in murine models. With IV injection of Ad-hFIX, outbred CD-1 mice uniformly produced anti-hFIX antibodies, whereas C57BL/6 hemopholic mice did not. Lympocytes from C57BL/6 animals treated with Ad-hFIX and multiply immunized with hFIX in complete Freund’s adjuvant did not proliferate when challenged with hFIX in contrast to the brisk responses observed in the BALB/c background. We initially compared expression of the intracellular reporter luciferase or of secreted hFIX in inbred C57BL/6, BALB/c, or CD-1 strains after AAV-8-mediated delivery, and did not detect differences in the level or duration of gene expression. Thus, initial neonatal studies were carried out in BALB/c mice, where transplantation and adoptive transfer studies are feasible. BALB/c neonates (n = 25) injected IP with 1.6 × 10^10 vg per g of AAV8-CBA-hFIX (AAV8-hFIX) on day 2 of life exhibited high level, stable expression of hFIX (Figure 1a) without induction of anti-hFIX or anti-AAV8 capsid antibodies (Figures 1a, c).

Augmenting hFIX levels by secondary IP administration of AAV8-hFIX is only successful in animals first injected at day 1–2 of life. While durable gene expression following a single administration of AAV has been observed in many studies, the immune responses to AAV capsid in adults has limited the ability to re-administer AAV and thereby boost gene expression levels. To test whether delivery of AAV early in immune ontogeny, during the first 4 weeks of life, enables re-administration of the same AAV serotype, BALB/c mice underwent primary injection with 1.6 × 10^9 vg per g.
of AAV8-hF.IX at different ages (day 1–2 of life, 1, 2 or 3 weeks of age and in adults). Levels of hF.IX were quantified by ELISA 4 weeks after primary injection, and serially thereafter for up to 40 weeks. As shown in Figures 3a–e, hF.IX levels after primary injection and prior to re-administration correlate directly with the number of vector genomes administered rather than with the stage of development, suggesting that transduction efficiencies were similar at these different time points. Nine weeks after primary injection, all groups were re-injected with 3.2 × 10^9 vg of AAV8-hF.IX. Only the group receiving primary injection on day 1–2 or day 7 (Figure 3a–e). Importantly, the augmented levels of hF.IX expression observed in mice treated at day 1–2, and again 9 weeks later, were stable for >40 weeks, suggesting that re-administration of the same AAV serotype did not break tolerance (Figure 3a).

To confirm that persistence of hF.IX expression after primary and secondary AAV delivery was predicated on the absence of anti-hF.IX humoral responses, plasma samples were screened before and after re-administration of AAV8-hF.IX in all mice. No anti-hF.IX antibodies were detected in experimental animals 8 weeks after primary injection, or 4 weeks after secondary injection of AAV8-hF.IX in any group (Figures 3f, g). All groups were then challenged with injection of hF.IX/Alum 17 weeks after secondary AAV8-hF.IX administration. ELISA assays did not detect anti-hF.IX antibodies in any of the AAV8-hF.IX-injected mice, consistent with the induction of immune tolerance to hF.IX in all groups (Figure 3h). Consequently, challenge with hF.IX/Alum did not affect the levels of hF.IX in mice from any of the groups.

There is a critical period in first week of life when IP injection of AAV8-hF.IX does not elicit humoral responses to AAV8 capsid protein. The observation that secondary injection of AAV8-hF.IX only augmented hF.IX expression in mice initially exposed to the vector as neonates suggested that immune responses to vector antigens limited the efficacy of re-administered vector. To test this hypothesis, serial plasma samples from the experimental groups described above were tested for the presence of anti-AAV8 antibodies. As hypothesized, the results of ELISA assays showed induction of anti-AAV8 antibodies in mice injected with AAV8-hF.IX after the first week of life, but not in animals initially injected at day 1–2 or day 7 (Figure 3a). Highest levels of anti-AAV8 antibodies, comparable to those in positive controls, were detected in mice injected at 3 weeks or as adults (Figure 3a). A similar pattern of anti-AAV8 capsid responses was observed in parallel experiments with delivery of AAV8-luciferase (data not shown). Thus, induction of tolerance to the expressed protein, and immunity to vector capsid, with neonatal delivery are not
AAV-hF.IX antigen-specific. Regardless of when the AAV8-hF.IX vector was administered, anti-AAV8 antibody production did not affect the initial levels of hF.IX expression.

The absence of anti-AAV8 capsid antibodies in the groups injected at day 1-2 or day 7 could either be due to the induction of tolerance, immune ignorance or to ineffective induction of B-cell
responses during the first week of life. To distinguish these possibilities, plasma samples were assayed for anti-AAV8 antibodies after secondary injection of AAV8-hF.IX in all of the experimental groups. As expected, high anti-AAV8 titers, comparable to positive controls, were observed in mice initially treated at 2 weeks and thereafter. Mice from neonatal and 1 week primary injection groups showed intermediate levels of anti-AAV8 antibodies indicating that tolerance to the transiently expressed AAV capsid antigens is not induced by vector administration during the first week of life (Figure 4b). Thus, the presence or absence of anti-AAV8 antibody following primary administration of AAV8-h.F.IX determines the efficacy of subsequent vector re-administration.

Adoptive transfer of splenocyte populations from mice neonatally injected with AAV8-h.F.IX specifically suppresses immune responses to h-F.IX

As there is evidence that CD4\(^+\)CD25\(^+\) Tregs have a major role in tolerance induction in adults after in vivo F.IX transfer,\(^{36,43}\) the potential role of antigen-specific Tregs in this neonatal model was explored. Splenocyte populations from naive or AAV8-h.F.IX neonatally injected BALB/c mice were adoptively transferred into naive BALB/c mice. Mice were then challenged by IP injection of h.F.IX in Alum 24 h after receiving unfractionated splenocytes, or after transfaction of purified lymphocyte subpopulations. Plasma samples were analyzed for anti-h.F.IX antibodies 2 weeks after h.F.IX/Alum administration. After adoptive transfer, mice transfected with unfractionated splenocytes from vector-treated animals produced, on average, two- to three-fold lower anti-h.F.IX antibody levels than control cohorts (Figure 5a). Adoptive transfer of purified CD4\(^+\) T cells (1 \times 10^7 cells) or CD4\(^+\)CD25\(^+\) cells (1 \times 10^6 cells) resulted in similar suppression of anti-h.F.IX humoral responses (Figure 5a). Transplantation of CD4-depleted splenocytes (CD4\(^-\) cells) (5 \times 10^7 cells) or CD4\(^-\)CD25\(^-\) T cells (9 \times 10^6 cells) failed to transfer tolerance, as the antibody titers measured were comparable to those detected in mice transfected with splenocytes from naive age-matched controls (Figure 5a). To test the antigen specificity of tolerance induction, and the ability of experimental animals to respond to a third party antigen, BALB/c mice neonatally injected with AAV8-h.F.IX vector were challenged by IP administration of recombinant luciferase protein formulated in Alum. These mice produced anti-luciferase antibody levels comparable to those in luciferase/Alum injected age-matched positive control mice (data not shown). The effect of adoptive transfer on immune responses to third party antigen was also tested by injection of luciferase/Alum 24 h after adoptive transfer of CD4\(^+\) T cells or CD4\(^-\) T cells from mice neonatally injected with AAV8-h.F.IX. ELISA for anti-luciferase antibodies showed no effect of adoptive transfer of CD4\(^+\) T cells on the humoral response of recipient BALB/c mice to luciferase (Figure 5b), confirming the integrity of immune responses in adoptively transplanted mice.

Phenotypic correction of hemophilia B mice in CD-1 mice is achieved with neonatal IP delivery of AAV-8 F.IX

To assess tolerance induction and therapeutic efficacy in a F.IX null background with robust immune responses, hemophilic mice were bred onto the outbred CD-1 background. In this murine model of hemophilia B, affected males and females express <1% levels of murine F.IX.\(^{29}\) CD-1 hemophilic B pups (n = 20) were injected at 1–2 days of life with 3.2 \times 10^9 v/g per g AAV8-h.F.IX. Sustained, therapeutic levels (400–600 ng/ml or 8–12% of normal human F.IX levels (5000 ng/ml) were detected (Figure 6a), comparable to F.IX levels measured in AAV8-h.F.IX-treated normal CD-1 mice (data not shown). Neither anti-h.F.IX nor anti-AAV8 antibodies were detected in serially collected plasma samples in hemophilic animals (Figures 6b, c). Correction of coagulopathy, demonstrated by correction of aPTT in AAV8-h.F.IX-treated hemophilic mice, (Figure 6c) resulted in a longer average lifespan (1.5 years vs 8 months in untreated mice).

Tissue distribution analysis of neonatal mice IP injected with AAV8-h.F.IX

In previous in vivo bioluminescence studies with IP AAV administration, we demonstrated that the majority of transduction and AAV-mediated gene expression was in the peritoneal lining of the abdomen and not in visceral organs, including the liver and spleen.\(^{44}\) We performed a tissue analysis to assess the sites of AAV8-h.F.IX transduction after IP delivery in neonates. The bio-distribution of AAV8-h.F.IX or AAV1-Luciferase vector genomes was assessed in adults 20 weeks after neonatal IP injection with 5 \times 10^9 v/g per g of either vector. Results of real-time PCR analysis of multiple tissues demonstrated that AAV-8 vector sequences are located primarily in the tissue samples from diaphragm and peritoneal wall (Figure 7), with little vector detected from liver or spleen. Parallel RT-PCR studies of mRNA from these tissues were consistent with the above studies showing highest levels of F.IX mRNA expression in tissues lining the peritoneal cavity, including diaphragm and peritoneum.
Other intra-abdominal and distal organs (heart) showed h.F.IX expression, albeit at lower levels. Thus, the majority of AAV8-hF.IX mRNA appears to reside in tissues lining the peritoneal cavity, indicating IP injection primarily mediates transduction of these tissues rather than entry of AAV into the systemic circulation, consistent with our previous studies.44

**DISCUSSION**

In these studies, intraperitoneal delivery of AAV8-hF.IX in neonates in the first month of life produced dose-dependent, stable levels of hF.IX expression. No humoral responses to hF.IX were detected after primary delivery of AAV8-hF.IX in the dose range administered, or after challenge with hF.IX protein and adjuvant. Adoptive transfer and in vivo depletion studies demonstrated that tolerance is mediated early in immune ontogeny, at least in part, by the induction of antigen-specific CD4+CD25+Tregs. Other F.IX gene transfer studies have focused on IM or IH delivery of AAV in adults.42,45 However, IM injections have been more likely to induce humoral responses than have IV or IH injections, even early in immune ontogeny.24 In studies by others, neonatal IP administration of AAV5-CMV-hF.IX produced stable, albeit lower...
levels of h.F IX than were achieved here with AAV8-hF.IX. In contrast to our IP delivery studies showing stable expression of the secreted hF.IX protein for >1 year, decreasing F.IX expression was observed in other neonatal models. Anti-hF.IX antibody responses described by others after IM AAV2-hF.IX injection, in utero or in neonates, were not elicited in the current study after IP injection of AAV8-hF.IX. As the continuous presence of self-antigens has been shown to be required for maintenance of tolerance in prior studies with neonates, it is likely that the stable hF.IX expression produced after neonatal IP administration is critical for the induction and maintenance of tolerance in this model.

We demonstrate a direct dose/response relationship between increasing doses of AAV8-hF.IX and hF.IX expression levels; however, induction of tolerance to hF.IX was not correlated with either the dose of AAV8-hF.IX in the dose range examined, or with levels of F.IX achieved after neonatal IP administration. This is in contrast to recent findings with delivery of AAV1-hF.IX in adult mice where induction of tolerance to hF.IX was AAV vector dose-dependent. In studies by Kelly et al., the minimum AAV1-hF.IX dose reproducibly resulting in tolerance induction in the C57BL/6 strain was 2 × 10^9 vg per g. Recent in utero IP gene delivery studies in fetal sheep using self-complementary AAV-hF.IX vectors (10 × 10^9 vg per g) also did not induce anti-hF.IX antibodies after primary injection; however, low levels of hF.IX were detected up to 6 months after injection with failure to induce tolerance to hF.IX. In our studies, tolerance to hF.IX was induced in all groups of animals, where levels of hF.IX expression were in general 20-fold higher at 6 months, than that achieved in the sheep model. Thus, not only the route of delivery and stage of immune ontogeny at which AAV is administered are critical in determining the level and nature of immune responses, but also whether sustained transgene expression levels are adequate for the maintenance of tolerance.

In this context, the immune mechanisms enabling long-term gene expression and absence of anti-capsid responses after neonatal delivery, and the time frame when this may be achieved have not been previously examined. In order to define such a critical period, primary injection at different ages (days 1–2 of life, 1, 2 or 3 weeks and adult) was tested and showed that only the group receiving days 1–2 injections exhibited a major increase in hF.IX after secondary injection. Consistent with this result, anti-AAV capsid antibody was not detectable in mice undergoing primary injection during the first week of life, but anti-AAV8 antibodies were detected in all other groups of mice after re-administration of AAV8-hF.IX.

A number of recent studies have addressed the challenges of establishing safe and effective routes of gene delivery, and reducing immune responses to AAV vectors in adults. Transvenular administration of F.IX in a canine model produced therapeutic levels of F.IX, albeit with anti-AAV capsid responses. Addition of transient immunosuppression enhanced F.IX levels and abrogated anti-F.IX antibody responses. In other studies, administration of proteasome inhibitors enhanced AAV-mediated transduction and decreased capsid antigen presentation. Transient immunosuppression with Mycophenolate Mofetil (MMF) and sirolimus or B-cell depletion with anti-CD20 have also reduced immune responses to AAV-mediated gene expression. Finally the use of micro-RNA-regulated gene therapy (miR-142 targeted) has been combined with the use of tissue-specific promoters to enhance cell specificity of expression and generate tolerogenic responses. The development of safe immunomodulatory approaches will enable more effective AAV-mediated genetic correction of hemophilia B.

Adoptive transfer of splenocyte populations demonstrated that CD4^+ CD25^+ cells from BALB/c mice, neonatally injected with AAV8-hF.IX, suppressed anti-hF.IX responses after hF.IX/Alum challenge. Administration of anti-CD25 antibody was used to deplete activated T cells, including T_{reg} transiently expressing CD25, that suppress effector T-cell responses necessary for the formation of anti-hF.IX antibody. Anti-CD25 antibody-mediated in vivo depletion of CD25^+ cells in mice tolerated by neonatal IP injection enabled induction of low-level anti-hF.IX antibody responses after hF.IX/Alum challenge.

The role of T_{reg} in specifically inhibiting immune responses and in the maintenance of self-tolerance has been investigated in several other contexts. In adult models of AAV-mediated F.IX gene transfer, conflicting data regarding the role of T_{reg} has been reported after hepatic versus IM administration. Suppression of antibody formation to hF.IX was shown to be mediated by CD4^+ CD25^+ T_{reg} after IH AAV-mediated hF.IX gene transfer. In contrast, with IM delivery of AAV1-hF.IX gene transfer, no increase in T_{reg} was detected in F.IX-tolerant mice and adoptive transfer of splenocytes from hF.IX-tolerant mice did not suppress anti-hF.IX immunity. Neither in vitro, nor in vivo depletion of T_{reg} reversed F.IX tolerance in this setting. Our data are consistent with the findings of Cao et al. with IH AAV-F.IX gene transfer in adult models, and support a major role for CD4^+ CD25^+ T_{reg} in tolerance induction to hF.IX after neonatal IP gene delivery.
The development of alternative strategies to achieve long-term transgene expression while avoiding toxic and abrogative immune responses represents an important and timely objective for genetic therapy. The relevance of these studies is underscored by pre-clinical studies in non-human primates in which codon-optimized, self-complementary AAV5 and AAV8-FIX were delivered IV-producing efficient hepatic transduction, but also stimulating serotype-specific humoral responses. Further defining immune mechanisms underlying tolerance induction during immune ontogeny may inform the design of novel approaches for limiting or eliminating immune responses that abrogate therapeutic gene expression.

MATERIALS AND METHODS

Animal care and procedures

BALB/c and CD-1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Hemophilia B F.IX KO mice were previously provided by Dr Inder Verma (The Scripps Research Institute, La Jolla, CA, USA).26 Mice were maintained in pathogen-free facilities, and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California, San Francisco (UCSF). Timed matings were established and vaginal plug dates recorded as day 1 of gestation. Dr Hengjun Chao60 (Mt Sinai School of Medicine, New York, NY, USA) at a dilution of 1:1000. Wells were blocked with 5% BSA in PBS. ELISA of anti-hF.IX and anti-AAV1 or AAV8 antibodies

Positive control adult antibodies were produced in adult mice by IP injection of 10 μg purified hFIX (CSL, Behring LLC) and Alum (Plurone) or by injection of 5 × 106 vg per g AAV virus and Alum and collected 4 weeks after immunization. For generation of a standard curve, serial dilutions of mouse anti-hFIX IgG (Haematologics Technologies Inc., Essex Junction, VT, USA) were applied to microtiter plates coated with purified hFIX protein (2.5 μg ml⁻¹—50 μg per well) starting at 100 ng ml⁻¹. Anti-hFIX was detected with goat anti-mouse IgG antibody conjugated with HRP in 1:1000 dilution (Calbiochem, San Diego, CA, USA). Negative controls for the anti-hFIX assay included plasma from AAV-luc virus/Alum-injected mice.

ELISA for detection of anti-AAV capsid antibodies utilized 2 × 106 vg virus particles coated on microtiter plates overnight at 4 °C. Mouse plasma samples were applied at a 1:200 dilution. Anti-AAV was detected with goat anti-mouse IgG antibody conjugated with HRP in 1:1000 dilutions (Calbiochem). Antibody levels were measured by OD reading at 450 nm following incubation with TMB. Negative controls for the anti-AAV assay included plasma from hFIX/Alum injected mice.

Adoptive splenocyte transfer

BALB/c mice neonatally injected with AAV8-hFIX were euthanized and splenocytes isolated. Total splenocytes were pooled and injected IV (4 × 107 cells in PBS/recipient naive BALB/c mouse, n = 5 per experimental group). Recipient mice were challenged with IP injection of 5 μg purified hFIX/Alum, 24 h after adoptive transfer. Anti-hFIX responses were measured 14 days later. For adoptive transfer experiments, CD4⁺ T cells, CD4⁻ T cells, CD4⁺ CD25⁻ T cells and CD4⁻ CD25⁻ T cells were purified from pooled splenocytes by magnetic cell sorting (Miltenyi Biotech, Auburn, CA, USA), according to manufacturer’s instructions. Briefly, CD4⁺ cells were isolated by negative selection using a combination of biotin-conjugated monoclonal antibodies against CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119, as well as anti-biotin MicroBeads (Miltenyi Biotech). CD4⁺ populations were isolated by depletion of CD4⁺ cells using mouse CD4 (LST4) MicroBeads. CD4⁺ CD25⁻ and CD4⁺ CD25⁺ populations were isolated using a CD4⁺ CD25⁻ Regulatory T Cell Isolation Kit (Miltenyi Biotech). FACs analysis showed >90% purity of isolated CD4⁺ splenocyte populations (data not shown). CD4⁺ T cells (4.5 × 10⁶ cells), CD4⁻ T cells (1.7 × 10⁷ cells), CD4⁺ CD25⁻ T cells (1.9 × 10⁶ cells) and CD4⁻ CD25⁻ T cells (2.75 × 10⁶ cells) were adoptively transferred to naive BALB/c mice (each group, n = 5) followed by challenge with hFIX/Alum 24 h later.

Activated partial thromboplastin time (APTPT) assay

The APTPT assay was carried out using SCA2000 veterinary coagulation analyzer (Symbiotics, San Diego, CA, USA) according to manufacturer’s instruction. The initial APTPT assay was performed 12 weeks after AAV8-CBA-hFIX administration and serially thereafter.

Biodistribution analysis

After neonatal IP administration of AAV8-hFIX, mice were euthanized at 20 weeks of age for tissue harvesting. DNA was isolated from all tissues using the QiaAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). PCR was performed with 200 ng of genomic DNA with primers described above for titering rAAV-hFIX. PCR was performed in triplicate using AAV8-hFIX plasmid as a standard. Gene copy number was calculated as copy number per microgram DNA.

RT-PCR analysis of hFIX mRNA expression in mice after IP AAV8-hFIX administration

Mice were euthanized 10 weeks after a single IP neonatal injection. Tissues were harvested and total RNA was extracted using RNeasy Mini Kit (Qiagen). RT-PCR was conducted using one-step RT-PCR kit (Qiagen). The first primer was 5'-TCCACTCGCTTCTGTTG-3', and 3' primer: 5'-CAGTGCGCA CGAGTTCA-3'. One-step RT-PCR was performed using the following conditions with 100 ng of total RNA per tissue sample: RT 50 °C, 30 min, PCR: 95 °C, 30 s; 58 °C, 45 s, 72 °C, 1 min for 30 cycles.
Statistical analysis
The data was analyzed using Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical differences between the various experimental groups were evaluated by t-test. P < 0.05 was considered statistically significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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