OPEN

Gene Therapy (2013) **20**, 987–996
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www.nature.com/gt

ORIGINAL ARTICLE

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

Y Shi, R Falahati, J Zhang, L Flebbe-Rehwaldt and KML Gaensler

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* $CD4^+CD25^+$ 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_{regs} 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.

Gene Therapy (2013) **20,** 987–996; doi:10.1038/gt.2013.22; published online 13 June 2013

Keywords: AAV; hemophilia B; immuno-tolerance; T_{reg}; neonatal

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.[1–3] Recent advances in 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.[4–9] 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,[10] and intravenous (IV) injection of AAV2-F.IX genomes cross-packaged into AAV-2/1, -2/4 and -2/6 vectors.[11] AAV1-mediated F.IX delivery also improved correction of hF.IX levels in large animal models of hemophilia B.[12] 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[13,14] AAV8-F.IX vectors also showed superior hepatic transduction with intraportal delivery in mice and non-human primates.[15,16]

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.[17] Studies by Nayak *et al.*[18] 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,[19] and high level F.IX expression was observed after transduction of hematopoietic stem cells with lenti-vectors expressing F.IX.[20] To address concerns regarding insertional mutagenesis, hepatocyte-targeted integrase-deficient lenti-vectors (IDLV) have been tested and produced robust F.IX gene expression without induction of neutralizing antibody responses in hemophilic mice.[21]

More recently, the role of innate inflammatory responses affecting lentiviral transduction and F.IX gene expression has been examined.[22] 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) as 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

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Received 25 May 2011; revised 7 January 2013; accepted 8 February 2013; published online 13 June 2013

evoke immune responses in murine models.[15,23,24] 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.[25,26] 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.[27–29] Retroviral-mediated delivery of the canine F.IX gene in newborn mice and dogs also directed therapeutic levels of F.IX expression,[30] but humoral responses to F.IX were detected with subsequent protein injections.[31] Injection of a variety of AAV serotypes in neonatal models does not elicit F.IX antibody responses.[24,32,33] 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 1).[34,35]

Studies with intra-hepatic AAV-F.IX delivery in adult transgenic models have defined the role of antigen-specific T regulatory cells (T_{reg}) in the successful induction of immune tolerance.[36] In adults, $CD4^+CD25^+$ T_{regs} represent 5–12% of $CD4^+$ T cells[16] and include both quiescent populations, and cells that continuously replicate in the presence of tissue self-antigens.[37] An important distinction between mice, as compared with sheep, non-human primates models and humans, is that mice are not immunologically competent at birth, while human fetuses achieve immune competence in the end of the second trimester.[38] In mice, T_{regs} do not begin to leave the thymus until day 3 after birth.[39] In this context, the role of T_{regs} in the induction of tolerance to viral gene delivery vectors and expressed therapeutic proteins early in immune ontogeny, has not been previously examined. The goals of the current studies were to determine whether there is a critical period, during which immune tolerance to both therapeutic genes and vectors might be achieved, and to define mechanisms underlying tolerance induction early in immune ontogeny.

RESULTS

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

Strain-specific differences in the immune responses to gene delivery vectors and hF.IX have been demonstrated in murine models. With IV injection of Ad-hF.IX, outbred CD-1 mice uniformly produced anti-hF.IX antibodies, whereas C57BL/6 hemophilic mice did not[40]. Lymphocytes from C57BL/6 animals treated with Ad-hFIX and multiply immunized with hF.IX in complete Freund's adjuvant did not proliferate when challenged with hF.IX in contrast to the brisk responses observed in the BALB/c background.[41] We initially compared expression of the intracellular reporter luciferase or of secreted hF.IX 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, inital 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-hF.IX (AAV8-hF.IX) on day 2 of life exhibited high level, stable expression of hF.IX (Figure 1a) without induction of anti-hF.IX or anti-AAV8 capsid antibodies (Figures 1a, c).

Dose escalation of AAV8-hF.IX administration in neonates produces increasing levels of hF.IX without induction of a humoral response

To determine whether there was a correlation between the level of circulating hF.IX and induction of humoral responses, as reported with adult IM AAV-hF.IX vector delivery,[42] BALB/c neonates were injected IP with increasing doses of AAV8-hF.IX ($1.6–32 \times 10^9$ vg per g) (each group, $n = 6$). A direct dose/response relationship was demonstrated between increasing doses of AAV8-hF.IX in this dose range, and hF.IX expression levels in neonates (Figure 2a). However, regardless of the dose of AAV8 or levels of hF.IX measured, no anti-hF.IX or anti-AAV8 capsid antibodies were detected in any of the neonatally injected groups (Figures 2b, c). Stable levels of gene expression were observed in animals injected at day 1–2 of life, despite the growth of animals and associated dilution of AAV. In adult animals injected IP with 1.6×10^9 vg per g AAV8-hF.IX ($n = 7$), the level and stability of hF.IX expression were comparable to that of the neonatally treated group receiving the same total viral dose (32×10^9 vg per g per animal). Antibody to AAV8 capsid was only detected in animals receiving IP injection of AAV8-hF.IX as adults (Figures 2b, c). Thus, the levels of hF.IX expression in all neonatal groups were sufficient to tolerize mice, even at the lowest dose of 1.6×10^9 vg per g.

Augmenting hF.IX levels by secondary IP administration of AAV8-hF.IX 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.[24] 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

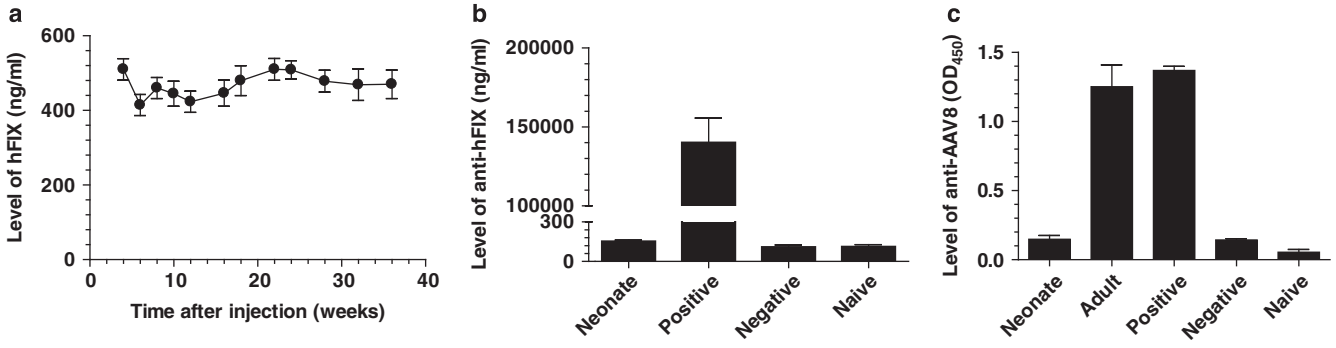

Figure 1. Long term, therapeutic hF.IX levels without induction of humoral responses after IP delivery of AAV8-hF.IX. BALB/c mice received IP injection of 1.6×10^{10} vg per g AAV8-hF.IX at 1–2 day of life ($n = 25$). Blood samples were collected beginning 4 weeks after AAV8-hF.IX delivery. (**a**) Human F.IX levels in plasma were quantified by ELISA. (**b**) ELISA of anti-hF.IX antibody titers. Positive control adults were produced by IP injection of 10 μg purified hF.IX/Alum. Negative controls included plasma from AAV8-Luc IP injected mice. Naive control plasma samples were from uninjected adult mice. (**c**) ELISA for anti-AAV8 capsid antibody. Positive control samples were produced by IP injection of adult mice with 5×10^9 Vg per g AAV8-Luc virus/Alum. The negative control for anti-AAV assay was the plasma from hF.IX/Alum-injected mice. ELISA of plasma samples from naive animals is also shown.

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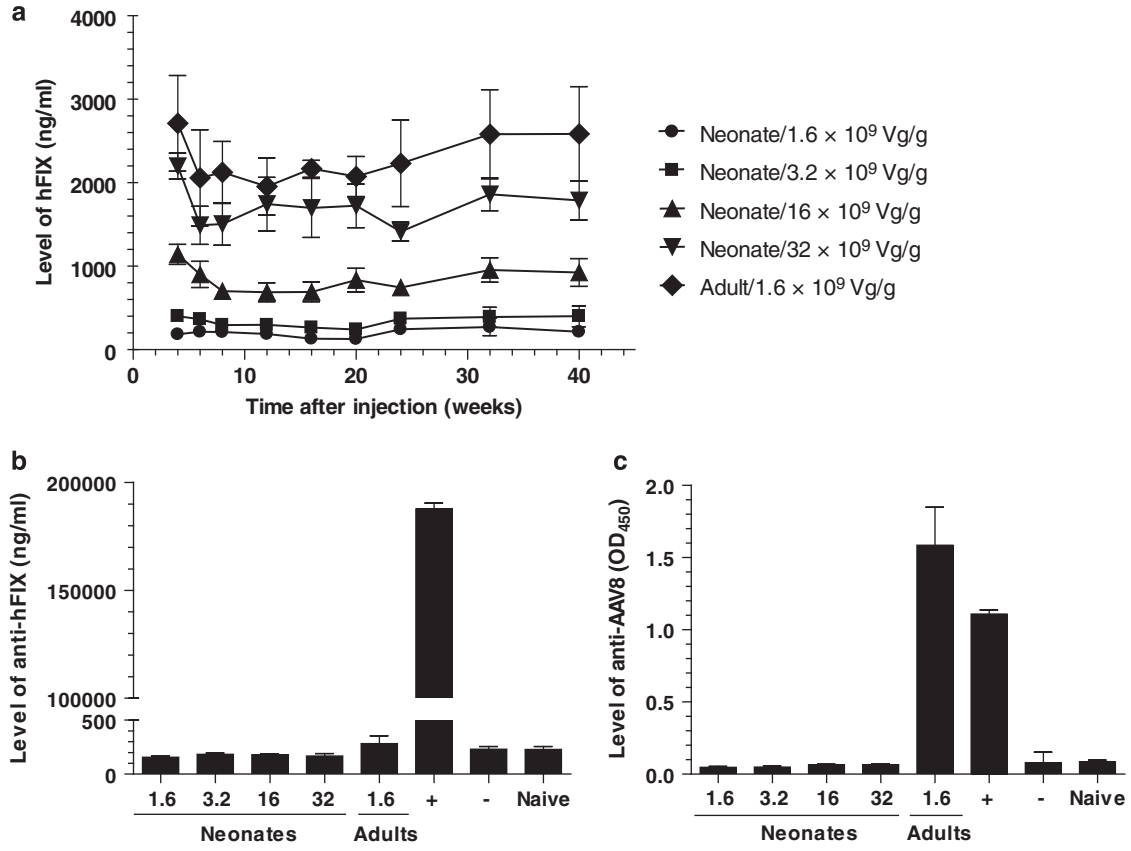

Figure 2. Dose-dependent hF.IX expression after neonatal IP administration of AAV8-hF.IX without induction of anti-hF.IX antibodies. After IP injection of BALB/c neonates with increasing dosages of AAV8-hF.IX ($1.6–32 \times 10^9$ vg per g) and adults at 1.6×10^9 vg per g, serial blood samples were collected beginning 4 weeks after AAV8-hF.IX delivery. (**a**) Human F.IX levels in plasma were quantified by ELISA as described. (**b**) ELISA of anti-hF.IX antibody titers after different IP doses of AAV8-hF.IX. The viral dose $\times 10^9$ vg per g is indicated for each group under individual columns. Samples from injected neonates or adults are indicated. Positive control (+) was plasma from hF.IX/Alum injected adults. Negative control (−) was plasma from AAV8-Luc injected mice. ELISA of plasma from naive animals is also shown. (**c**) ELISA for anti-AAV8 capsid antibody. Experimental groups are the same as described in Figure 2b. Positive control (+) was plasma from adult mice injected with 5×10^9 vg per g AAV8-Luc/Alum. Negative control (−) was plasma from hF.IX/alum-injected animals. ELISA of plasma from naive mice is shown at far right.

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 IP with 3.2×10^{10} vg of AAV8-hF.IX. Only the group receiving primary injection on day 1–2 exhibited a major increase in hF.IX with secondary injection (Figure 3a). A smaller increment in hF.IX was observed in mice initially injected at 1 week of age. No significant boost in expression was observed in any of the mice receiving primary injections after 1 week of age (Figures 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

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 4a). Highest levels of anti-AAV8 antibodies, comparable to those in positive controls, were detected in mice injected at 3 weeks or as adults (Figure 4a). 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

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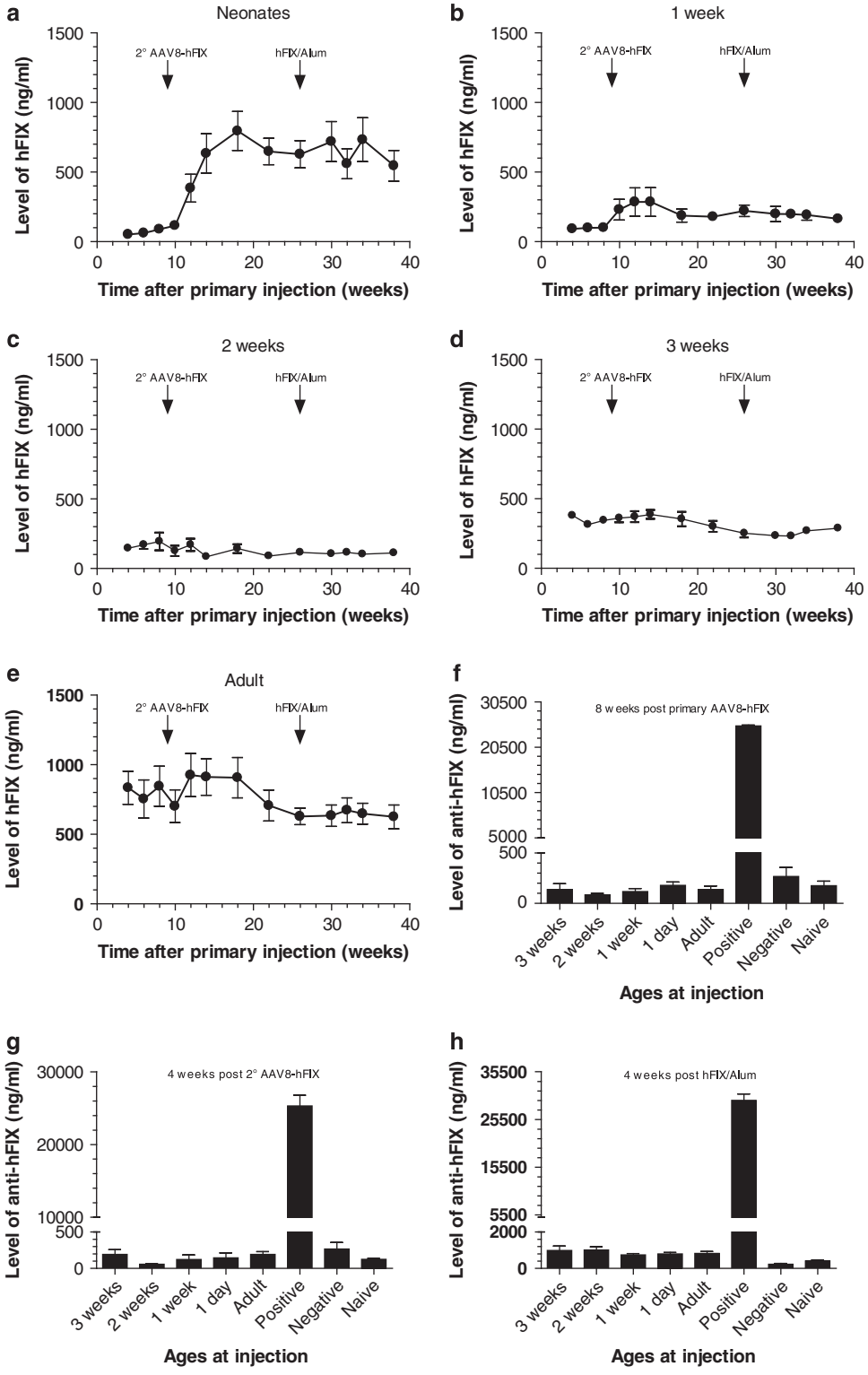

Figure 3. Augmented hF.IX levels with AAV8-hF.IX re-administration only observed in animals receiving primary injection at day 2 of life. BALB/c mice received primary injection with 1.6×10^9 vg per g of AAV8-hF.IX at the indicated ages (1–2 day of life, 1, 2 3 weeks or adult). Secondary IP injection of AAV8-hF.IX was performed 9 weeks after the primary injection in all groups. All groups were then challenged with injection of hF.IX/Alum 17 weeks after secondary AAV8-hF.IX administration. (**a**–**e**) hF.IX levels were initially quantified in plasma by ELISA, 4 weeks after primary injection, and serially thereafter up to 40 weeks. (**f**–**h**) anti-hF.IX antibody levels. (**f**) 8 weeks after primary injection; (**g**) 4 weeks after secondary AAV8-hF.IX IP injection; (**h**) 4 weeks after challenge with hF.IX/Alum. The age of mice at primary AAV8-hF.IX injection is indicated below each column. Positive (+), Negative (−) and naive controls are as described in Figure 2b.

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

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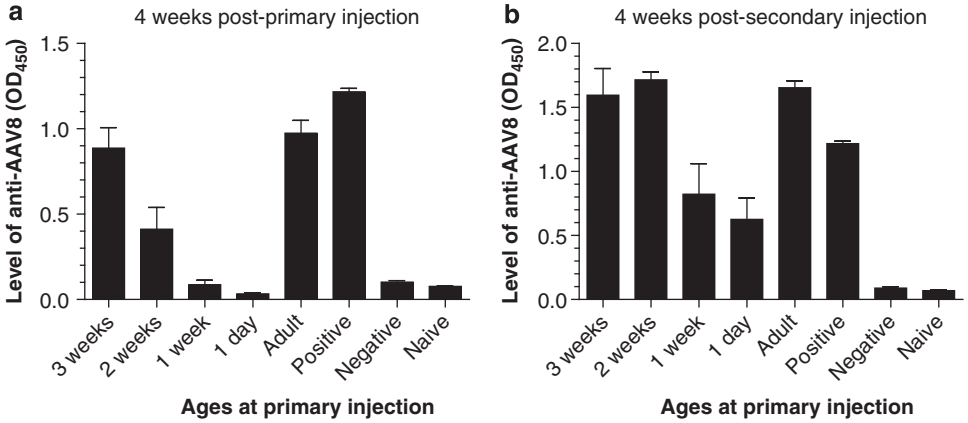

Figure 4. Critical period for IP AAV administration without anti- AAV8 capsid after IP injection of AAV-hF.IX. Primary IP injection of 1.6×10^9 vg per g of AAV8-hF.IX in BALB/c mice at different ages (1–2 day of life, 1, 2 3 weeks of life or as adults) was followed by re-administration of AAV8-hF.IX 9 weeks later. Serial blood samples were collected beginning 4 weeks after primary AAV8-hF.IX delivery for ELISA to detect anti-AAV8 capsid antibodies. Positive, negative and naive controls were as described in Figures 1c. (**a**) Anti-AAV8 capsid titers 4 weeks after primary injection (**b**) Anti-AAV8 capsid titers 4 weeks after secondary injection of AAV8-hF.IX (13 weeks after primary injection).

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-hF.IX determines the efficacy of subsequent vector re-administration.

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

As there is evidence that $CD4^+CD25^+$ T_{regs} have a major role in tolerance induction in adults after *in vivo* F.IX transfer,[36,43] the potential role of antigen-specific T_{regs} in this neonatal model was explored. Splenocyte populations from naive or AAV8-hF.IX neonatally injected BALB/c mice were adoptively transplanted into naive BALB/c adults. Mice were then challenged by IP injection of hF.IX in Alum 24 h after receiving unfractionated splenocytes, or after transplantation of purified lymphocyte subpopulations. Plasma samples were analyzed for anti-hF.IX antibodies 2 weeks after hF.IX/Alum administration. After adoptive transfer, mice transplanted with unfractionated splenocytes from vector-treated animals produced, on average, two- to three-fold lower anti-hF.IX antibody levels than control cohorts (Figure 5a). Adoptive transfer of purified $CD4^+$ T cells (1×10^7 cells) or $CD4^+CD25^+$ cells (1×10^6 cells) resulted in similar suppression of anti-hF.IX humoral responses (Figure 5a). Transplantation of CD4-depleted splenocytes ($CD4^-$ cells) (5×10^7 cells) or $CD4^+CD25^-$ T cells (9×10^6 cells) failed to transfer tolerance, as the antibody titers measured were comparable to those detected in mice transplanted 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-hF.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-hF.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 hemophilia B pups ($n = 20$) were injected at day 1–2 of life with 3.2×10^9 vg per g AAV8-hF.IX. Sustained, therapeutic levels (400–600 ng/ml or 8–12% of normal human hF.IX levels (5000 ng/ml) were detected (Figure 6a), comparable to hF.IX levels measured in AAV8-hF.IX-treated normal CD-1 mice (data not shown). Neither anti-hF.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-hF.IX-treated hemophilic mice, (Figure 6d) 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-hF.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-hF.IX transduction after IP delivery in neonates. The bio-distribution of AAV8-hF.IX or AAV1-Luciferase vector genomes was assessed in adults 20 weeks after neonatal IP injection with 5×10^9 vg 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 hF.IX mRNA expression in tissues lining the peritoneal cavity, including diaphragm and peritoneum

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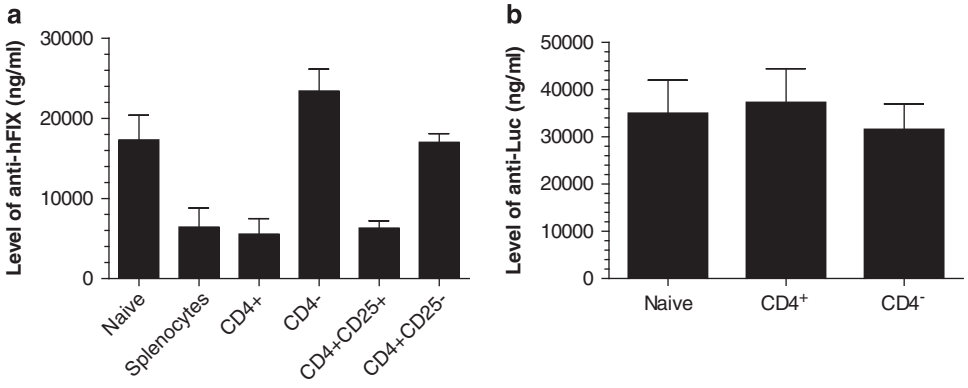

Figure 5. Effect of adoptive transfer of splenocyte populations from AAV8-hF.IX neonatal injected mice on immune responses to hF.IX/Alum and Luc/Alum challenge. Spenocytes from naive adult mice or adult mice after neonatal AAV8-hF.IX delivery, were infused IV, 24 h before challenge with hF.IX/Alum or Luc/Alum. Total splenocytes (5×10^7), purified $CD4^+$ T cells (1×10^7), $CD4^-$ cell (5×10^7), $CD4^+CD25^+$ cells (1×10^6) or $CD4^+CD25^-$ T cells (9×10^6) were transferred. Each bar represents the average antibody titer for 5 animals ($\pm$ s.d.). (**a**) Anti-hF.IX antibody titer. (**b**) The level of anti-Luc antibody 2 weeks after immunologic challenge. Response to third party antigen was tested by injection of luciferase/Alum 24 h after adoptive transfer of splenocyte populations, as described in (**a**).

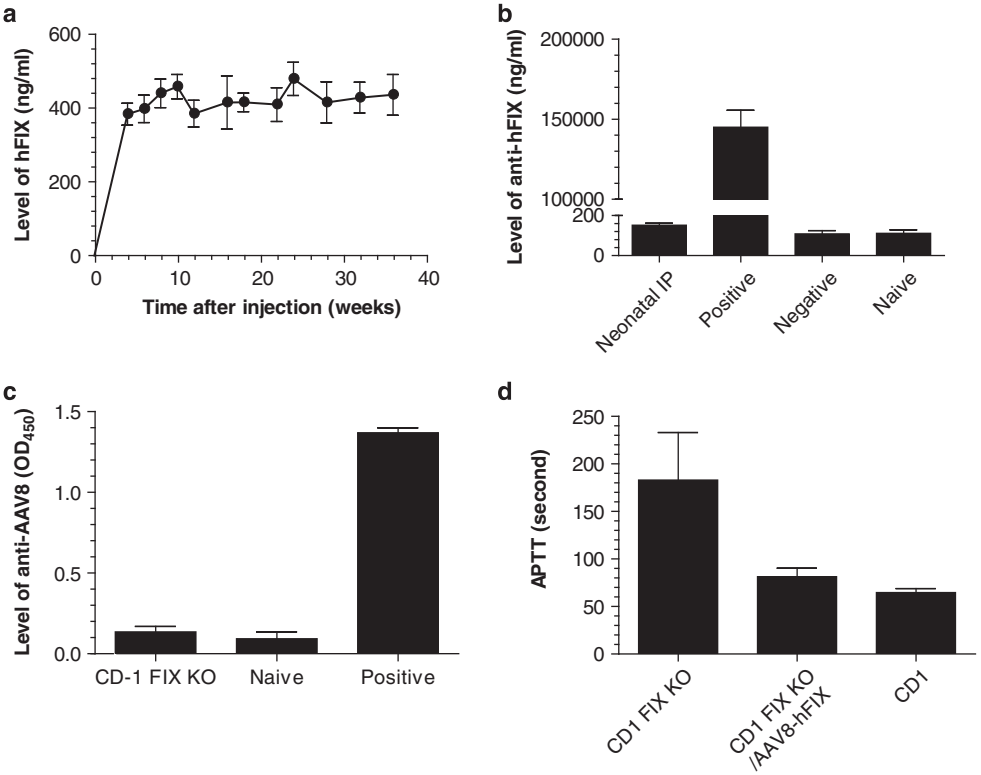

Figure 6. Phenotypic correction of F.IX knockout mice in CD-1 strain without induction of anti-hF.IX or anti-AAV8 capsid antibodies after delivery of AAV8-hF.IX on 1–2 day of life. Treatment of hemophilia B (CD-1 $F9^{-/-}$ designated as CD-1 FIX KO) mice by neonatal IP AAV8-hF.IX gene transfer (3.2×10^9 Vg per g) ($n = 10$). Shown are: (**a**) systemic hF.IX levels. (**b**) the level of anti-hF.IX antibodies 4 weeks after vector administration, (**c**) measurement of anti-AAV8 capsid antibody titers, and (**d**) measurement of coagulation time by aPTT, 4 weeks after IP vector administration.

(Figure 8). Other intra-abdominal and distal organs (heart) showed h.F.IX expression, albeit at lower levels. Thus, the majority of AAV8-F.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-F.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 $CD4^+CD25^+$ T_{regs}. 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

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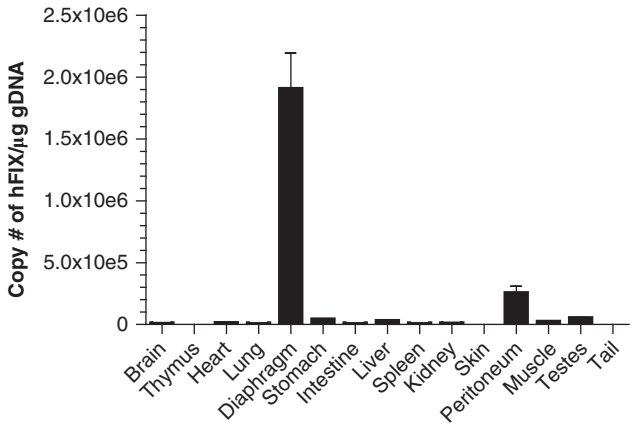

Figure 7. Tissue bio-distribution analysis of mice injected neonatally IP with AAV8-hF.IX in neonates. Mice were injected on day 1–2 of life doses of 5×10^9 vg per g for AAV8-hF.IX. Tissues were harvested from neonatally injected mice at 20 weeks after vector administration. Real-time PCR was performed for detecting the number of copies in 200 ng of genomic DNA, and the data is displayed as the number of hF.IX copies per μg gDNA. Negative controls are tissue from naive BALB/c mice.

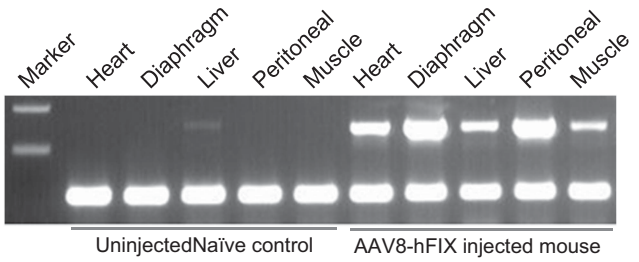

Figure 8. RT-PCR analysis of hF.IX mRNA expression in mice neonatally administrated with AAV8-hFIX IP. Mice were sacrificed 10 weeks after a single neonatal injection. Tissues were harvested and total RNA was extracted and amplified by RT-PCR. First lane on left: marker; Five lanes on left show RT-PCR products from an un-injected, age-matched, naive control mouse. The five lanes on the right represent RT-PCR products from tissues harvested after IP neonatal injection of AAV8-hF.IX. Tissues analyzed include heart, diaphragm, liver, muscle from lower extremity, and peritoneum with underlying muscle.

levels of hF.IX than were achieved here with AAV8-hF.IX.[32] 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.[46] Anti-hF.IX antibody responses described by others after IM AAV2-hF.IX injection, *in utero* or in neonates,[24] 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,[47,48] 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.[42] In studies by Kelly *et al.*[42] the minimum AAV1-hF.IX dose reproducibly resulting in tolerance induction in the C57BL/6 strain was 2×10^{10} 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.[49] 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-AAV8 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.[50] Addition of transient immunosuppression enhanced F.IX levels and abrogated anti-F.IX antibody responses.[51] In other studies, administration of proteasome inhibitors enhanced AAV-mediated transduction and decreased capsid antigen presentation.[52,53] Transient immunosuppression with Mycophenolate Mofetil (MMF) and sirolimus[54] or B-cell depletion with anti-CD20 have also reduced immune responses to AAV-mediated gene expression.[55] 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.[56] 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.[36] Anti-CD25 antibody-mediated *in vivo* depletion of $CD25^+$ cells in mice tolerized 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.[57,58] 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.[36,42,46] Suppression of antibody formation to hF.IX was shown to be mediated by $CD4^+CD25^+$ T_{regs} after IH AAV-mediated hF.IX gene transfer.[36] 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.[42] Neither *in vitro*, nor *in vivo* depletion of T_{regs} reversed F.IX tolerance in this setting.[42,46] Our data are consistent with the findings of Cao *et al.*[36] 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.

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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-F.IX were delivered IV-producing efficient hepatic transduction, but also stimulating serotype-specific humoral responses.[59] 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).[29] 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.

The hemophilia B knockout strain was bred for more that 15 generations onto the CD-1 outbred background. Animals were screened by PCR genotyping using primers pairs: 5′-AGCGAAGGAGCAAAGCTGCTATT-3′ and 5′-GTAGTGAATGTTGTGGACCTAAG-3′ to detect wild type, 5′-AGCGAAG GAGCAAAGCTGCTATT-3′ and 5′-AAGGAAGACACAGAACAAAGG-3′ for the F.IX KO allele. For studies in hemophilic mice, CD-1 offspring of heterozygous F.IX knockout females (X^hX) (F.IX-deleted X chromosome designated as X^h) and hemophilic F.IX-affected males (X^hY) were injected IP with 3.2×10^{10} vg per g AAV8-hF.IX on day 1–2 of life ($n = 10$). Genotyping by PCR was carried out on pups 2 weeks after injection.

Virus delivery

BALB/c normal mice were initially injected intra-peritoneally (IP) with indicated doses of AAV8-hF.IX at day 1–2 of life, or at different ages (1 week, 2 weeks and 3 weeks). Immunizations were performed by intra-peritoneal injection of 10 μg purified human F.IX (CSL, Behring LLC, Kankakee, IL, USA) formulated in Alum (Pierce, Rockford, IL, USA) or by injection of 5×10^9 vg per g AAV virus/Alum to generate anti-hF.IX or anti-AAV antibody responses, respectively.

Preparation of recombinant AAV vectors

Recombinant AAV viruses were prepared by triple-plasmid co-transfection as described previously.[44] Two AAV helper plasmids, pAV8h and pAV1h provided by Dr Roland W Herzog (University of Florida, Gainesville, FL, USA) were used to produce rAAV8-hF.IX and rAAV1-hF.IX. Vector plasmid pCAhF9 is an expression vector of the human coagulation factor IX (hF.IX) gene controlled by the CBA promoter kindly provided by Dr Hengjun Chao[60] (Mt Sinai School of Medicine, New York, NY, USA). 293T cells were harvested 48 h after transfection, and virus isolated and purified by cesium chloride gradient centrifugation. Virus preparations were titered by real-time PCR (RT-PCR) using primer pair of 5′-CCTCAGTACAGGAGGCAAACC-3′, reverse 5′-GATGGAGATCAGTGTGAGTCC-3′ targeting hF.IX sequences. A viral standard curve was based on the serially diluted pCAhF9 plasmid solution.

Measurement of hF.IX level in blood

Concentrations of hF.IX in mouse plasma samples were determined using enzyme-linked immunosorbent assay (ELISA) using human plasma as a standard.[40] Briefly, 96-well plates were coated overnight at 4 °C with a monoclonal anti-hF.IX antibody (clone HFIX-1; Sigma-Aldrich, St Louis, MO, USA) at a dilution of 1:1000. Wells were blocked with 5% BSA in PBS. Mouse plasma samples were diluted with 5% BSA in PBS to concentrations within the linear range of the standard curve. A polyclonal goat anti-h.FIX antibody conjugated with horseradish peroxidase (HRP) (Affinity Biologicals, Hamilton, Ontario, Canada) was used as the secondary antibody in a dilution of 1:1000. After applying TMB substrate (Neogen, Lexington, KY, USA), the 96-well plate was read at a single wavelength of 445 nm. Levels of hF.IX were calculated from a standard curve derived from serial dilutions of pooled normal human plasma (Sigma-Aldrich) diluted in normal isogenic mouse plasma.

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 hF.IX (CSL, Behring LLC) and Alum (Pierce) or by injection of 5×10^9 vg per g AAV virus and Alum and collected 4 weeks after immunization. For generation of a standard curve, serial dilutions of mouse anti-hF.IX IgG (Haematologic Technologies Inc., Essex Junction, VT, USA) were applied to microtiter plates coated with purified hF.IX protein ($2.5\ \mu g\ ml^{-1}$—50 μl per well) starting at $100\ ng\ ml^{-1}$. Anti-hF.IX 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-hF.IX assay included plasma from AAV-luc virus/Alum-injected mice.

ELISA for detection of anti-AAV capsid antibodies utilized 2×10^8 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 hF.IX/Alum injected mice.

Adoptive splenocyte transfer

BALB/c mice neonatally injected with AAV8-hF.IX were euthanized and splenocytes isolated. Total splenocytes were pooled and injected IV (4×10^7 cells in PBS/recipient naive BALB/C mouse, $n = 5$ per experimental group). Recipient mice were challenged with IP injection of 5 μg purified hF.IX/Alum, 24 h after adoptive transfer. Anti-hF.IX 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 (L3T4) 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 (4.5×10^6 cells), $CD4^-$ T cells (1.7×10^7 cells), $CD4^+CD25^+$ T cells (1.9×10^6 cells) and $CD4^+CD25^-$ T cells (2.75×10^7 cells) were adoptively transferred to naive BALB/c mice (each group, $n = 5$) followed by challenge with hF.IX/Alum 24 h later.

Activated partial thromboplastin time (APTT) assay

The APTT assay was carried out using SCA2000 veterinary coagulation analyzer (Synbiotics, San Diego, CA, USA) according to manufacturer's instruction. The initial APTT assay was performed 12 weeks after AAV8-CBA-hF.IX administration and serially thereafter.

Biodistribution analysis

After neonatal IP administration of AAV8-hF.IX, mice were euthanized at 20 weeks of age for tissue harvesting. DNA was isolated from all tissues using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). PCR was performed with 200 ng of genomic DNA with primers described above for titering rAAV-hF.IX. PCR was performed in triplicate using AAV8-hF.IX 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 5′ primer was: 5′-TCACCATCTGCCTTTTAGG-3′, and 3′ primer: 5′-CAGTGGGCA GCAGTTACA-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.

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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.

ACKNOWLEDGEMENTS

We thank Dr Inder Verma for originally providing FIX KO mice (Scripps Research Institute, La Jolla, CA, USA), Dr Roland W Herzog for providing plasmids pAV8h and pAV1h, (University of Florida, Gainesville, FL, USA), and Dr Hengjun Chao for providing hF.IX plasmid (Mt Sinai School of Medicine). We also appreciate constructive suggestions and discussion with Dr. William J. Murphy (University of California, Davis, CA, USA). This work was supported by NIH grant R01DK072537 to KMLG.

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