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Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response

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We have previously shown that a single portal vein infusion of a recombinant adeno-associated viral vector (rAAV) expressing canine Factor IX (F.IX) resulted in long-term expression of therapeutic levels of F.IX in dogs with severe hemophilia B[1]. We carried out a phase 1/2 dose-escalation clinical study to extend this approach to humans with severe hemophilia B. rAAV-2 vector expressing human F.IX was infused through the hepatic artery into seven subjects. The data show that: (i) vector infusion at doses up to 2×10^{12} vg/kg was not associated with acute or long-lasting toxicity; (ii) therapeutic levels of F.IX were achieved at the highest dose tested; (iii) duration of expression at therapeutic levels was limited to a period of ~8 weeks; (iv) a gradual decline in F.IX was accompanied by a transient asymptomatic elevation of liver transaminases that resolved without treatment. Further studies suggested that destruction of transduced hepatocytes by cell-mediated immunity targeting antigens of the AAV capsid caused both the decline in F.IX and the transient transaminitis. We conclude that rAAV-2 vectors can transduce human hepatocytes *in vivo* to result in therapeutically relevant levels of F.IX, but that future studies in humans may require immunomodulation to achieve long-term expression.

Hemophilia B is a bleeding diathesis caused by mutations in the gene encoding blood coagulation F.IX. The majority of individuals with hemophilia B have severe disease, with <1% normal circulating levels of F.IX activity; improvement of levels into the range of only ~5% results in a profound improvement in symptoms and is sufficient to prevent spontaneous and life-threatening bleeding episodes[2,3]. Moreover, preclinical studies[1,4,5] have shown that long-lasting expression of such levels can readily be achieved in the canine model of hemophilia B by a single infusion of rAAV–canine F.IX vector (rAAV-F.IX) into the portal vein. In an earlier study, we documented that rAAV-F.IX delivered to skeletal muscle of individuals with severe hemophilia B was safe, but circulating levels were generally not sufficient to improve disease phenotype[6,7]. Based on the safety studies of rAAV-F.IX delivered to muscle in humans and the efficacy studies of rAAV-F.IX delivered to liver in dogs with severe hemophilia B, we undertook an open-label, dose-escalation study of rAAV-F.IX (**Fig. 1a**) delivered through the hepatic artery in humans with severe hemophilia B (**Table 1**). We found that, in contrast to long-lasting expression in hemophilic dogs and nonhuman primates, expression at therapeutic levels in humans was short lived. A series of follow-up experiments indicated that in humans, T cell–mediated immunity to AAV capsid antigens causes destruction of AAV-2–transduced hepatocytes, leading us to conclude that rAAV-2 can successfully transduce human hepatocytes *in vivo*, but that a modification of the protocol will be required to achieve long-lasting expression in humans.

After informed consent, adult males (**Table 2**) with severe (<1% F.IX activity) hemophilia B who met all other enrollment criteria

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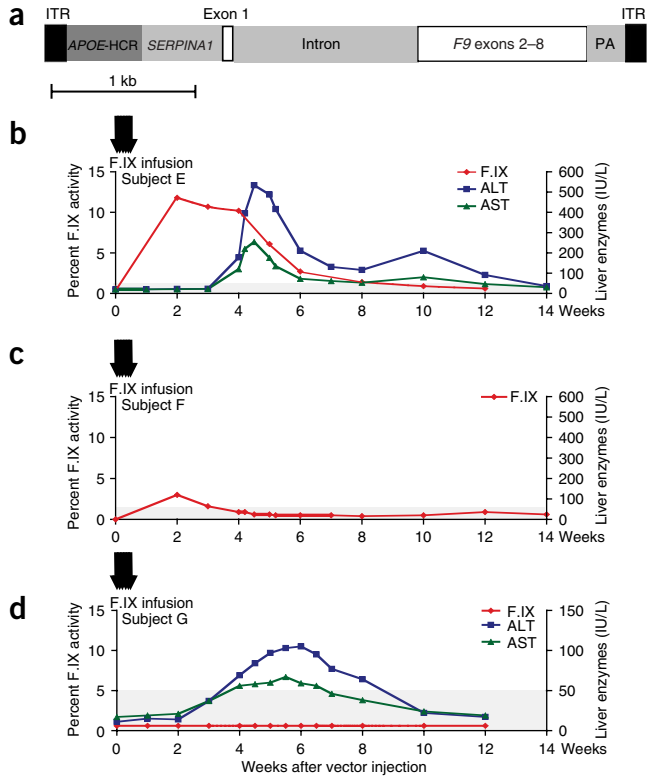

Figure 1 *F9* expression cassette and results of rAAV-F.IX in subjects E, F and G. (**a**) A schematic of the *F9* (encoding F.IX) expression plasmid used to prepare the recombinant AAV vector. The human *SERPINA1* (α_1-antitrypsin, also known as hAAT) promoter is linked to the hepatic locus control region and a single copy of the *APOE* enhancer (*APOE*-HCR). The human *F9* cDNA sequence is interrupted by a 1.4 kb fragment of the first intron, as previously described[24]. PA, polyadenylation signal; ITR, viral inverted terminal repeat. (**b–d**) F.IX activity assay and transaminase levels (AST, ALT) plotted as a function of time in weeks after vector administration in subjects E, F and G. (**b**) Data for subject E, who received 2×10^{12} vg/kg rAAV-F.IX. Arrows at upper left denote days when F.IX protein concentrate was infused. (**c**) Data for subject F, infused at same dose as subject E. Shaded gray panel denotes normal range for AST, ALT. (**d**) Data for subject G, infused at 4×10^{11} vg/kg. The scale for transaminases is expanded compared to **b** and **c**, to show the increase in levels first noted at week 3.

(**Supplementary Table 1** online) were admitted to one of the three participating medical centers. After baseline studies, subjects received F.IX protein-replacement product, and were then infused through the hepatic artery (**Supplementary Methods** online) with rAAV-F.IX (**Fig. 1a**). None of the subjects experienced adverse reactions (for example, fever, chills, nausea or vomiting) after vector infusion. Three subjects developed small hematomas at the site of catheter insertion in the groin. One 63-year-old subject (subject A) died of a myocardial infarction 12 months after vector infusion. The death was judged unrelated to the vector infusion (see **Supplementary Note** online).

Clinical laboratory studies including complete blood counts, coagulation parameters, liver function tests and blood urea nitrogen–creatinine were performed at regular intervals (**Table 2**). No abnormalities were noted in any subjects for the first 3 weeks after vector infusion (data not shown). Two subjects developed elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) beginning 4 weeks after vector infusion. Otherwise there was no evidence of toxicity associated with vector infusion. We conclude that infusion of rAAV-F.IX at doses up to 2×10^{12} vg/kg is not associated with any acute toxicity in men with severe hemophilia B, including individuals with a history of hepatitis C infection.

A PCR assay was developed to detect vector sequences in body fluids; spiking experiments showed reliable detection of as few as 100 copies of plasmid or vector DNA in 1 μg human DNA. Results from serum and urine (**Supplementary Table 2** online) suggested that time to clearing (PCR signal from the vector is no longer detectable) was dose dependent. Urine was the first body fluid to clear. There was no association between dose and time to clearing for semen. The last positive semen samples in the lowest dose cohort were at 10 weeks and 12 weeks, whereas the last positive samples in the five subjects in the two higher-dose cohorts occurred at a mean of 4.8 weeks. Age of the subject seemed to be the best predictor of time to clearing of semen, with young men on average clearing more quickly than older men. Semen fractionation, carried out for a single subject, showed no evidence of vector sequences in motile sperm (**Supplementary Table 3** online). Peripheral blood mononuclear cells (PBMCs) showed the longest duration of positivity of any body fluid or tissue analyzed.

The first four subjects (subjects A, B, C and D), two in each of the initial dose cohorts, showed no evidence of vector-related toxicity or of efficacy, defined as circulating F.IX levels of >1%. Data from subjects E, F and G were more informative (**Fig. 1b–d**). Subject E, the first to be infused in the third dose cohort, had a pretreatment neutralizing antibody (NAB) titer to AAV of 1:2. He was HCV antibody positive and RNA viral load negative. He underwent vector infusion uneventfully; 2 weeks later, 1 week after the last infusion of F.IX concentrate, his F.IX activity level was 11.8%. The F.IX level persisted in this range until 5 weeks after infusion, when it declined to 6.1%. The level continued to fall gradually over the next 5 weeks, reaching the baseline of <1% 10 weeks after vector infusion (**Fig. 1b**). F.IX antigen assays performed by ELISA at weeks 2–5 documented antigen levels in close agreement with activity assays (**Supplementary Table 4** online). Clinically, the subject reported an absence of any bleeding episodes during these 10 weeks, and there was no infusion of clotting factor, despite trauma which would ordinarily have required factor infusion. In contrast to previously infused subjects, this subject manifested an asymptomatic rise in transaminases beginning 4 weeks after vector infusion. Aminotransferases (ALT, AST) were within normal limits at baseline and for the first 3 weeks after vector infusion. The peak level of ALT (532 IU/L) was measured 32 d after vector infusion, with a gradual return to normal levels in the ensuing 9 weeks (**Fig. 1b**). There was no evidence of a F.IX inhibitor at any time point. A F.IX fall-off curve (to measure the half-life of infused F.IX) performed 3 years after vector administration was essentially identical to a fall-off curve carried out 10 years earlier (data not shown).

Subject F received the same dose of vector as subject E, but his pretreatment NAB titer to AAV-2 was higher, at 1:17. The infusion and immediate postinfusion period were uneventful, and laboratory

Table 1 Dose-escalation plan for clinical trial and results of same doses in experimental animals

Group	Subjects	Dose/kg (vg)	Total dose[a] (vg)	Factor IX levels in mice	Factor IX levels in dogs
1	2	8.0×10^{10}	5.6×10^{12}	~1%	0.5–1%
2	3	4.0×10^{11}	2.8×10^{13}	5%	5–10%[b,c]
3	2	2.0×10^{12}	1.4×10^{14}	20–30%	5–10%[b,c]

[a]Assuming a 70 kg subject. [b]Results achieved using dose of 1.0×10^{12} vg/kg, intermediate between groups 2 and 3. [c]Levels were achieved using construct similar but not identical to construct used in the clinical trial[7].

Table 2 Trial subject demographics and results

	Group 1		Group 2			Group 3	
	8×10^{10} vg/kg		4×10^{11} vg/kg			2×10^{12} vg/kg	
	A	B	C	D	G	E	F
Age (years)	63	48	21	20	27	31	28
Baseline F.IX activity	<1%	<1%	<1%	<1%	<1%	<1%	<1%
Mutation	R16Stop	W310 Stop	[a]	R180 Q	W407Stop	G133E	R-4Q[c]
CRM status	Neg	Neg	N/A	Pos	Neg	Neg	Pos
HIV Ab	Neg	Pos	Neg	Pos	Neg	Neg	Neg
HCV Ab	Pos	Pos	Pos	Pos	Neg	Pos	Pos
HCV RNA	Neg	Pos	Pos	Pos	Neg	Neg	Neg
HB_s Ag/Ab	–/+	–/–	–/+	–/–	–/+	–/+	+/–
Liver biopsy	N/D	F1[b]	F0[b]	F1[b]	N/D	N/D	N/D
Follow-up (months)	12	40	34	33	15	31	30
LFTs	WNL	WNL	WNL	WNL	Transaminitis < Grade 1	Transaminitis Grade 3	WNL
F.IX inhibitor	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Max F.IX assay	<1%	<1%	<1%	<1%	<1%	11%	3%
Serious adverse events	Acute MI Expired 12 mos. post vector infusion	None	None	None	None	None	None
Pre-treatment AAV antibody titer	N/A	1:2	1:2	1:11	<1:2	1:2	1:17

[a]No mutation found in the coding region. [b]Metavir score[28,29]. [c]Mutation in propeptide, position –4. CRM, cross-reacting material; Ab, antibody; Ag, antigen; N/A, not available; N/D, not indicated and not performed; LFT, liver function test; WNL, within normal limits; MI, myocardial infarction.

studies were all within normal limits. Two weeks after infusion, the F.IX level was 3%, and had returned to the baseline of <1% by 4 weeks (**Fig. 1c**). This subject was also hepatitis C antibody positive and RNA viral load negative, but did not develop an elevation in transaminase levels (data not shown).

As had been documented previously in the AAV-F.IX muscle trial[6,7], infusion of vector was followed by a several log increase in neutralizing antibody titer to AAV in all subjects (**Supplementary Table 5** online). No antibodies to F.IX were ever detected in any subject (**Table 2**).

The asymptomatic transaminitis that began 4 weeks after vector infusion in subject E had never been observed previously in animals infused with AAV-F.IX. Possible causes of the increase in transaminase levels included toxic or infectious causes of liver injury, direct hepatocellular injury from the vector, or an immune response to some component of the vector. Infectious causes of transaminitis were sought and excluded (**Supplementary Table 6** online), and direct hepatocellular injury from vector seemed unlikely, as the transaminitis had not been seen in animals and was first observed in this human subject beginning 4 weeks after vector infusion. The time course suggested an immune-mediated destruction of transduced hepatocytes, which could explain both the increase in transaminase levels and the coincident decline of circulating F.IX. The original clinical protocol had not included collection of PBMCs for detailed studies of the immune response, but was subsequently modified to allow this. At the request of the regulatory agencies, the next subject studied was infused at a dose of 4×10^{11} vg/kg, a dose that had been documented to be subtherapeutic in two previous subjects (C and D). Subject G had a low pretreatment NAB titer to AAV-2, and no history of hepatitis virus infection. He tolerated vector infusion uneventfully and showed no changes in laboratory studies until week 3 after vector infusion when transaminases began to increase, although they initially remained within normal limits. By week 4, both AST and ALT were above the upper limit of normal; values peaked ~6 weeks after vector infusion, with a maximum ALT concentration of 105 IU/L and AST concentration of 67 IU/L (**Fig. 1d**). The subject remained asymptomatic during this period. As had been documented previously at this vector dose, circulating F.IX levels did not increase to >1%.

The presence of NAB to AAV pretreatment in humans seems to modulate the linear dose response seen in animals. Thus subjects E and F received the same dose but only subject E, with low pretreatment antibodies, achieved any appreciable level of transduction. Similarly, among subjects C, D and G, who all received the same vector dose, only subject G, with the lowest pretreatment NAB to AAV-2, showed any change in transaminases. In the two subjects with the lowest pretreatment NAB, subjects E and G, the height of the ALT spike was directly proportional to the dose of vector received.

Transduction by AAV-F.IX presents two foreign antigens to the hemophilic subject's immune system: the AAV capsid and wild-type F.IX. To screen for T-cell responses to these antigens, we used ELISPOT assays for interferon (IFN)-γ or interleukin (IL)-5 secretion after stimulation of subject G's PBMCs with overlapping peptide matrices spanning the AAV-2 VP1 capsid protein and wild-type F.IX (**Fig. 2**). The ELISPOT data showed no detectable AAV capsid–specific response before treatment, but IFN-γ secretion after stimulation with three peptide pools was detected 2 weeks after vector infusion (first time point assayed) and remained positive at weeks 5 and 8, with a decline to levels below the detection limit by week 12 (**Fig. 2**). Positive peptide pools 2, 10 and 19 map the response to peptides 74 and 82 in the AAV-2 capsid (**Fig. 2b,c**); stimulation of subject PBMCs with the isolated peptide 74 also elicited robust secretion of IFN-γ (**Fig. 2a**), whereas stimulation with peptide 82 elicited more modest secretion of IFN-γ (data not shown). ELISPOT assays for IL-5 were consistently negative, and there was no IFN-γ secretion in response to F.IX peptides (data not shown). We carried out high-resolution human leukocyte antigen (HLA) genotyping on subject G (data not shown); we then used two online bioinformatics tools, RANKPEP and nHLAPred, to identify peptide sequences within the AAV-2 capsid that had a high predicted binding potential to the subject's major histocompatibility complex (MHC) class I molecules. In both programs, a subsequence of peptide 74 (VPQYGYLTL) had the highest binding potential within the capsid

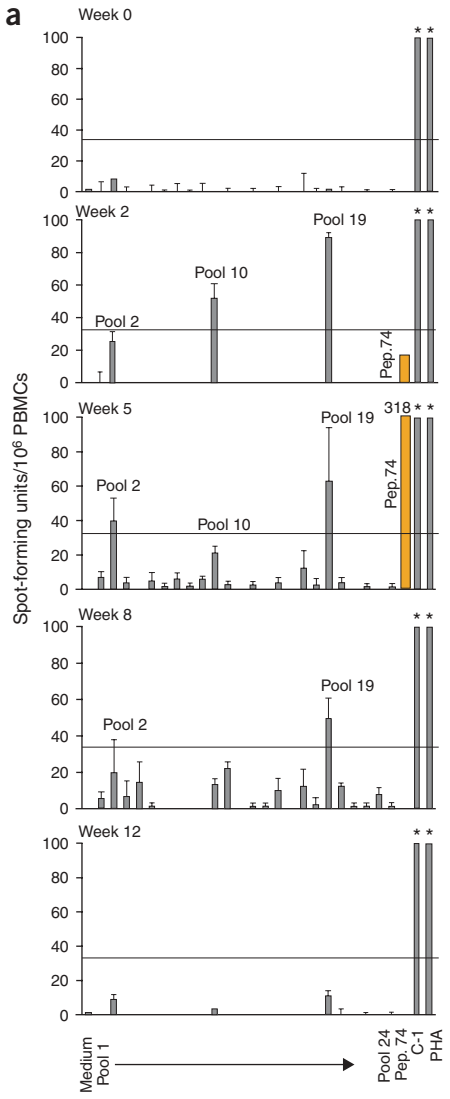

Figure 2 IFN-γ ELISPOT data in subject G. (**a**) Secretion of IFN-γ from the PBMCs of subject G after stimulation with 24 pools of peptides derived from AAV-2 capsid sequence. PBMCs were drawn at the indicated time points ranging from before infusion to 12 weeks after vector infusion. The *x*-axis shows (left to right) medium (negative control), pools 1–24, peptide 74 alone (orange bars, tested only at weeks 2 and 5), control peptide pool 1 (C-1, a collection of peptides derived from viruses common in the population) and phytohemagglutinin (PHA; positive controls). The red horizontal line denotes the limit for a positive readout in this assay. Asterisks indicate that spots are too numerous to count. (**b**) Matrix array of 145 peptides spanning the sequence of the AAV-2 VP-1 protein. Pool numbers are highlighted in blue. Positive pools are highlighted in yellow. Positivity in two orthogonal pools specifies a single stimulatory peptide (highlighted in orange). (**c**) Sequence of the two 15-mers identified by ELISPOT assay. These are highly conserved in AAV serotypes 1–8 (**Supplementary Fig. 1** online).

sequence for the subject's *B*0702* allele (data not shown). Peptide 82 was not identified using either of these bioinformatics tools. The data show that the only detectable T-cell response in PBMCs is to AAV capsid, not to F.IX.

Recombinant AAV vectors have provided long-term cures of genetic disease in a number of large-animal models[1,4,5,8–13]. The tropism for postmitotic cells of interest, stabilization as a predominantly nonintegrated form and the lack of strong inflammatory responses to AAV-mediated gene transfer are all attractive features of the vector. In the case of hemophilia B, the preclinical data in large-animal models are particularly convincing, with multiple groups showing long-term expression of F.IX at levels well within the therapeutic range (5–10%) after a single infusion of infusion of rAAV-F.IX into the portal vein[1,14,15].

Based on these preclinical findings, we conducted a clinical gene transfer trial of rAAV-F.IX to the liver in humans with hemophilia B. The data show that human liver can be transduced by rAAV after *in vivo* delivery of the vector, and that vector infusion is not accompanied by any acute toxicity. The study also documented therapeutic levels of F.IX, albeit only transiently, at the doses predicted to be therapeutic based on studies in hemophilic dogs[1], validating the dog as a model for dose determination at least for rAAV-2. But this study also uncovered problems not encountered in the preclinical studies in animals.

The study provides evidence that rAAV-2 can directly transduce human liver. The vector has a liver-restricted promoter and cannot be expressed to any appreciable extent in other tissues[16,17]. In subject E, the F.IX level was measured at >10% for 4 weeks after vector infusion, whereas the last dose of recombinant F.IX was infused 1 week after vector administration. Because the half-life of recombinant F.IX in this subject was documented to be 16–21 h, it is clear that the circulating levels measured during weeks 2, 3 and 4 must have arisen from the donated gene, not from infused protein. Though F.IX levels can be difficult to measure accurately in the range of 1%, there is no such difficulty at the higher levels seen in subject E. The agreement between antigen (**Supplementary Table 4** online) and activity levels, and the gradual decline in levels beginning 5 weeks after transduction, with all points falling along a physiologically convincing curve, are consistent with expression from the transgene.

An early concern in this trial was evidence of vector DNA in semen based on PCR analysis of semen DNA, raising the possibility of germline transmission. But this proved to be transient in all subjects, with younger subjects clearing more quickly than older ones. Moreover, animal studies suggest that rAAV-2 does not transduce spermatogonia directly[18,19], and semen fractionation studies in rabbits (V.R.A., unpublished data) and in one human subject (**Supplementary Table 3** online) are at least consistent with the notion that the vector is present in the seminal fluid but does not transduce motile sperm. In this study this potential complication was managed by recommending that: (i) all subjects bank sperm before enrollment; and (ii) subjects use barrier contraception until the semen was negative for vector sequences. A limitation of the findings is that, because tissue tropism is capsid dependent, these findings cannot be extrapolated to other AAV serotypes.

The other unexpected finding in this study was an asymptomatic transient elevation in transaminases, beginning several weeks after vector infusion, and noted in two subjects, E and G. A detailed study of these events supports the following hypothesis. First, it seems probable that the transaminitis arose from specific destruction of vector-transduced cells by the immune system. The temporal relationship between a detectable PBMC response to AAV capsid, declining F.IX levels and rising transaminases are all consistent with this hypothesis. Second, the T-cell studies on subject G suggest that AAV capsid, rather than wild-type F.IX, is the antigen that is targeted by the host immune response. The response shown by ELISPOT assays probably reflects at least in part the activity of $CD8^+$ T cells as indicated by recognition of an MHC class I binding peptide. It should be pointed out that AAV capsid antigens, unlike F.IX, are not synthesized in transduced cells. MHC class I presentation pathways favor peptides derived from *de novo* produced proteins; nevertheless, alternative pathways exist that allow for appropriate presentation of proteins passively taken up by cells. These apparently sufficed for recognition of AAV-transduced hepatocytes by $CD8^+$ effector T cells.

A logical extension of this hypothesis provides an explanation for the difference in outcome of AAV-mediated gene transfer to liver in humans compared to experimental animals, all of which, including mice, rats, hemophilic dogs and nonhuman primates, have shown long-term expression of the transgene after AAV-mediated gene transfer[1,4,14,15]. Prior exposure to the capsid probably underlies the difference in response. Unlike experimental animals, humans are naturally infected by AAV-2 during childhood. Because AAV is naturally replication defective, this initial infection invariably takes place together with a helper virus infection such as adenovirus. Although AAV-2 on its own may not induce inflammatory reactions needed for stimulation of a maximal adaptive immune response, in combination with the helper virus, which causes activation of the innate immune system, it is likely that $CD8^+$ T cells directed to the antigens of the helper virus and of AAV are formed. Upon controlling the infection, the frequency of AAV-specific $CD8^+$ T cells would be expected to decline, leaving behind a small pool of memory T cells, which through homeostatic proliferation are maintained throughout the life of an individual[20]. On reexposure to AAV capsid, these memory $CD8^+$ T cells are activated and eliminate the AAV capsid–harboring cells (the transduced hepatocytes). Because memory T cells are more readily triggered than naive lymphocytes, human subjects, undergoing reexposure, have a different outcome than experimental animals do, undergoing what amounts to primary infection with AAV. Consistent with this hypothesis, we observed a maximal IFN-γ ELISPOT response in PBMCs early on, 2 weeks after vector infusion; as the transduced cells are destroyed, the detectable response in PBMCs also disappears.

The studies reported here identified AAV capsid rather than F.IX as the offending antigen that triggered immune-mediated destruction of the transduced hepatocytes. A strategy based on an attempt to engineer out the offending capsid sequence is probably doomed to failure given the highly polymorphic nature of the human MHC loci. Differences in kinetics of vector uncoating among various AAV serotypes may influence the outcome[21,22], but switching serotypes may be of limited utility given the high degree of conservation of T-cell epitopes identified to date (**Supplementary Fig. 1** online). On the other hand, the capsid, needed to deliver the therapeutic gene, is present only transiently in the transduced cell, because it is not encoded in the vector and is gradually degraded and cleared. Thus a short-term immunomodulatory regimen that blocks the response to capsid until these sequences are completely cleared from the rAAV-2–transduced cells may permit long-term expression of the donated gene.

METHODS

Preparation of rAAV-hAAT-F.IX. rAAV-hAAT-F.IX (hAAT, human α_1-antitrypsin) vector was prepared using current good manufacturing practices. Vector preparation was by helper virus–free transient transfection of HEK-293 cells[6]; the F.IX expression plasmid was a 11,188 bp construct containing a liver-specific promoter composed of the human *SERPINA1* (α_1-antitrypsin) promoter, the *APOE* enhancer, and the hepatic control region, coupled to the human *F9* cDNA interrupted by a 1.4 kb fragment of intron 1, as previously described[16,17,23]. We purified the vector using density centrifugation as previously described[24] with minor modifications. We formulated the final purified vector in 10 mM sodium phosphate, 140 mM sodium chloride, 5% sorbitol, 0.1% polysorbate 80 (pH 7.3), sterile filtered it, and titered it using a previously described Q-PCR procedure[25].

Clinical study. The study was approved by the Institutional Review Boards (IRBs) at The Children's Hospital of Philadelphia, Stanford University Medical Center and the University of Pittsburgh Medical Center, where vector infusions took place, as well as by the IRBs of the hospitals of the hemophilia centers where subjects were followed. The study was also reviewed and approved by the US Food and Drug Administration and the US National Institutes of Health Recombinant DNA Advisory Committee, and was monitored by the US National Heart, Lung, and Blood Institute Data Safety Monitoring Board.

Demographic characteristics, F.IX mutation and preexisting viral infection profiles for the seven subjects are presented in **Table 2**. Prior infection with hepatitis virus B or C was not an exclusion criterion, nor was HIV infection or concurrent antiretroviral treatment. At the request of the regulatory agencies, each subject was observed for at least 4 weeks before the next subject was enrolled; thus, if two subjects in a dose cohort showed neither efficacy nor toxicity, the dose was increased in the next cohort.

Vector administration through hepatic artery infusion. Details of the angiographic procedure for vector infusion are available in **Supplementary Methods** online. Subjects were observed in the hospital for at least 24 h after vector infusion. F.IX protein was administered daily for 7 d, to prevent bleeding from the arterial puncture site.

Clinical laboratory assays. Unless otherwise specified, laboratory studies were performed in the clinical laboratories at the Children's Hospital of Philadelphia, Stanford University Medical Center or University of Pittsburgh Medical Center using Clinical Laboratory Improvement Amendments (CLIA)-approved procedures. F.IX activity assays were determined using an automated analyzer (MDA-180, Biomerieux). For additional details, see **Supplementary Methods** online.

Neutralizing antibody assays to AAV. AAV-2–specific NAB titers were determined as previously described[6]. Data are also reported using a four-parameter curve fit to determine the theoretical serum dilution for 50% inhibition of AAV transduction (IC_{50}). Values shown in **Supplementary Table 5** are from an average of four assays ± error of the mean ($1/IC_{50}$ ± s.d.). For all subjects, NAB titers to AAV-2 rose by logs after vector infusion (**Supplementary Table 5** online).

PCR assays on body fluids. Serum, urine, saliva and stool were collected at baseline, 24 h, 48 h, 1 week and then weekly after vector infusion until three consecutive samples were negative. In the most recent version of the protocol, semen and PBMCs were collected at baseline, 1 week, 4 weeks and then monthly until three consecutive samples were negative (earlier versions contained more frequent semen collections). We developed and validated a PCR assay for rAAV-F.IX to screen human samples for the presence of vector DNA (**Supplementary Methods** online).

ELISPOT assays for peptide-stimulated production of IFN-γ and IL-5 by PBMCs. PBMCs were collected using standard techniques and cryopreserved until assay[26]. Peptide libraries consisting of 15-mers overlapping by 10 amino acids with the adjacent peptide were prepared for the AAV-2 VP-1 capsid

sequence (735 amino acids, 145 peptides), and the F.IX sequence (461 amino acids, 91 peptides; GeneMed Synthesis or Mimotopes). Peptides were dissolved in 50% acetonitrile at a concentration of 3 mg/ml (stock solution), and arrayed in pools, each containing 12–13 peptides, such that the stimulatory peptide would be identified when two orthogonal pools were positive. ELISPOT assays were performed by the reference laboratory of the US National Institutes of Health Immune Tolerance Network (Cellular Technology, Ltd.). IFN-γ is secreted by $CD8^+$ and $CD4^+$ T helper type 1 (T_H1) cells, whereas IL-5 is secreted by $CD4^+$ T_H2 cells. We plated 3×10^5 PBMCs per well into a 96-well ELISPOT plate, and incubated them in the presence or absence of peptide (each peptide at 2 μg/ml) for 24 h at 37 °C. We tested pools in triplicate and repeated assays with relevant peptides when sufficient cells were available. Medium alone served as a negative control, and phytohemagglutinin as a positive control. In addition, the responses to 23 MHC class I–restricted peptides[28] that represent T-cell epitopes of viruses common in the human population (cytomegalovirus, Epstein-Barr virus, influenza), and able to bind to a broad range of HLA molecules served as positive controls. Results are shown as spot-forming units/10^6 cells. For additional details of ELISPOT assays, see **Supplementary Methods** online.

Bioinformatics programs. For details, see **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have competing financial interests (see the *Nature Medicine* website for details).

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ERRATUM: People to watch

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In the version of this article originally published, a sentence in the second paragraph reads: "These cells are one of only a few types of nerve cells that are known to regenerate." The correct version reads: "These cells are one of only a few types of nervous system cells that are known to promote regeneration."

CORRIGENDUM: Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response

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In the version of this article originally published, Pradip Rustagi was inadvertently omitted from the author list; John Rasko should be listed as John E. J. Rasko; the affiliations of John E. J. Rasko and Katherine A. High were incorrect; and the author order was incorrect. The correct list of authors with their affiliations is:

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The error has been corrected in the HTML and PDF versions of the article.