



REGULAR ARTICLE

# Immune response after neonatal transfer of a human factor IX-expressing retroviral vector in dogs, cats, and mice

Lingfei Xu<sup>a,b</sup>, Manxue Mei<sup>a,b</sup>, Mark E. Haskins<sup>c,d</sup>, Timothy C. Nichols<sup>e</sup>, Patricia O'Donnell<sup>c,d</sup>, Karyn Cullen<sup>c,d</sup>, Aaron Dillow<sup>e</sup>, Dwight Bellinger<sup>e</sup>, Katherine P. Ponder<sup>a,b,\*</sup>

<sup>a</sup> Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO, USA

<sup>b</sup> Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO, USA

<sup>c</sup> Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia PA, USA

<sup>d</sup> Department of Clinical Studies, University of Pennsylvania School of Veterinary Medicine, Philadelphia PA, USA

<sup>e</sup> Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA

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## Abstract

*Introduction:* Gene therapy could prevent bleeding in hemophilia. However, antibodies could inhibit coagulation, while cytotoxic T lymphocytes could destroy modified cells. The immaturity of the newborn immune system might prevent these immune responses from occurring after neonatal gene therapy.

*Materials and methods:* Newborn dogs, cats, or mice were injected intravenously with a retroviral vector expressing human Factor IX. Plasma was evaluated for antigen and anti-human Factor IX antibodies. Cytotoxic T lymphocyte responses were evaluated indirectly by analysis of retroviral vector RNA in liver. Lymphocytes were evaluated for cytokine secretion and the ability to suppress an immune response to human Factor IX in mice.

*Results and conclusions:* Hemophilia B dogs that achieved 942±500 ng/ml (19% normal) or 5±0.4 ng/ml (0.1% normal) of human Factor IX in plasma only bled 0 or 1.2 times per year, respectively, and were tolerant to infusion of human Factor IX. Normal cats expressed human Factor IX at 118±29 ng/ml (2% normal) in plasma without antibody formation. However, plasma human Factor IX disappeared at late times in 1

*Abbreviations:* FIX, Factor IX; IV, intravenous; hFIX, human FIX; RV, retroviral vector; MPS I, mucopolysaccharidosis I; CTL, cytotoxic T lymphocyte; cIDUA, canine  $\alpha$ -L-iduronidase; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; IU, international units; HGF, hepatocyte growth factor; IP, intraperitoneal; WBCT, whole blood clotting time.

\* Corresponding author. Department of Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA. Tel.: +1 314 362 5188; fax: +1 314 362 8813.

E-mail address: [kponder@im.wustl.edu](mailto:kponder@im.wustl.edu) (K.P. Ponder).

of 4 cats, which was probably due to a cytotoxic T lymphocyte response that destroyed cells with high expression. C3H mice were tolerant to human Factor IX after neonatal gene therapy, which may involve clonal deletion of human Factor IX-responsive cells. These data demonstrate that neonatal gene therapy does not induce antibodies to human Factor IX in dogs, cats, or mice. The putative cytotoxic T lymphocyte response in one cat requires further study.

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## Introduction

Hemophilia B is due to deficiency of Factor IX (FIX), occurs in 1:30,000 males, and is treated with intravenous (IV) injection of FIX protein [1]. However, ~5% of patients that receive protein replacement therapy develop antibodies, which block the function of FIX (inhibitors) or affect its half-life [2]. Antibodies can make bleeding episodes difficult and expensive to treat. Similarly, although gene therapy has successfully treated hemophilia B in small and large animal models [3–7], antibody responses can reduce the efficacy. Prevention of antibody development would be important for protein or gene therapy.

Several approaches have been used to prevent an immune response after gene therapy for hemophilia B [8]. One is to achieve high levels of expression, which reduces the chance of an immune response compared with low expression [9,10]. This is consistent with the practice of injecting high doses of human FIX (hFIX) to induce tolerance in patients with inhibitors [2]. Some have reported that liver-restricted expression of FIX reduced antibody responses, although this was not consistently effective [10,11]. Alternative approaches are to use immunosuppressive agents [8,12], to transduce hematopoietic stem cells [13], or to administer peptides intranasally [14]. Finally, fetal [15] and neonatal [16] gene transfer have induced tolerance to FIX in mice due to the immaturity of the immune system at the time of gene transfer.

In our previous report, hemophilia B mice on a C57BL/6 background that received IV injection of an hFIX-expressing gamma retroviral vector (RV) were tolerant to hFIX protein challenge if baseline plasma hFIX levels were above 14 ng/ml ( $3 \times 10^{-10}$  M) [16]. However, it was unclear if this would be effective in higher animals, which have a more-mature immune system at birth than mice [17]. This previous study also demonstrated that normal dogs that received neonatal gene transfer with a high dose of RV achieved 500 ng/ml of hFIX without an antibody response [16]. However, these dogs were not tested to determine if they were truly tolerant, and the effect of RV dose and hFIX expression on immune responses was not evaluated. In addition, it was

unclear if this would be effective in hemophilia B dogs, which have a missense mutation and very low plasma canine FIX levels [18,19]. Therefore, we extended these studies to evaluate the level of hFIX required to induce tolerance after neonatal gene transfer in normal and hemophilia B dogs.

Immune responses to hFIX were also evaluated in normal cats in this study. These experiments were initiated when we discovered that cats with mucopolysaccharidosis I (MPS I) mount a cytotoxic T lymphocyte (CTL) response to canine  $\alpha$ -L-iduronidase (cIDUA) after neonatal gene therapy with a cIDUA-expressing RV [20]. These data suggested that cats may have a more-mature immune system at birth than mice or dogs, and could provide a useful model in which to evaluate inhibitor or CTL responses to hFIX after gene transfer.

It will be important to understand the mechanism by which neonatal gene transfer induces tolerance. The immune system of many species, including humans, is immature at birth, although immune responses can be induced with strong stimuli [21]. Potential mechanisms of neonatal tolerance include clonal deletion, suppression, and ignorance [22]. The final goal of this study was to investigate the mechanism of neonatal tolerance to hFIX in mice.

## Materials and methods

### Reagents

Reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The RV designated hAAT-hFIX-WPRE contains the human  $\alpha_1$ -antitrypsin promoter, the hFIX cDNA, and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) [16]. Recombinant hFIX [BeneFix, specific activity 270 international units (IU)/mg] was generously supplied by Wyeth Pharmaceutical (Cambridge, MA). Hepatocyte growth factor (HGF) was purified as described [23].

### Animal procedures

All animals received care in compliance with the American Convention on Animal Care, and studies were approved by the institutional Ethics

Committee. Normal dogs [24] and cats [25] at the University of Pennsylvania were heterozygous for a lysosomal storage disease. Chapel Hill hemophilia B dogs [18] were housed at the University of North Carolina. At 2 to 5 days after birth, hAAT-hFIX-WPRE was injected IV into dogs or cats at the doses indicated in the figure legends. For protein challenge, hFIX was injected IV at 30 IU/kg. Plasma was anticoagulated with 1/10 volume of 3.2% sodium citrate.

Newborn C3H/HeJ (referred to here as C3H) mice from the Jackson Laboratory (Bar Harbor, ME) received IV injection at 2–3 days after birth with hAAT-hFIX-WPRE to give the dose indicated in the figure legends. For adult (6 week-old) mice, some received HGF given intraperitoneal (IP) with dextran sulfate every 3 h from 0 to 12 h for a cumulative dose of 25 mg/kg as described [26] to induce hepatocyte replication, and others received phosphate buffered saline (PBS; Mediatech, Inc., Herndon, VA) at the same times. All adult mice received hAAT-hFIX-WPRE IV at the cumulative dose indicated in the figure legends at 30 to 48 h after the first dose of HGF or PBS. For protein challenge, mice were injected IP with 30 IU/kg/dose of hFIX in 200  $\mu$ l of PBS or with the adjuvant RIBI MPL+TDM emulsion (Corixa Corporation, Hamilton, MT) as described [16]. Plasma was collected via a non-heparinized capillary tube from the retro-orbital plexus and mixed with 1/10 volume of 3.2% sodium citrate.

### hFIX antigen and antibody assays

The hFIX antigen immunoassay [16] used a mouse monoclonal anti-hFIX antibody (HIX-1, F2645) and a horseradish peroxidase (HRP)-conjugated goat anti-hFIX antibody (GAFIX-APHRP, Enzyme Research, South Bend, IN) and was sensitive to 0.5 ng/ml. To detect anti-hFIX antibodies, ELISA wells were coated with 5  $\mu$ g/ml of hFIX in PBS, and samples were diluted 1:100 or higher with 100 mM NaCl, 50 mM Tris pH 7.5, and 5% (gm/dl) carnation fat-free dry milk (Schnucks Grocery, St. Louis, MO). For samples from mice, the 2nd antibodies were HRP-conjugated goat anti-mouse total IgG, or anti-mouse IgG<sub>1</sub>, IgG<sub>2a</sub> or IgG<sub>2b</sub> antibodies (Roche Molecular Biochemicals, Indianapolis, IN). The standards were dilutions of purified total mouse IgG, or purified mouse IgG<sub>1</sub>, IgG<sub>2a</sub>, or IgG<sub>2b</sub>, respectively. For dog samples, HRP-conjugated sheep anti-canine total IgG (Serotec Inc., Raleigh NC), or sheep anti-canine IgG<sub>1</sub> or IgG<sub>2</sub> antibodies (Bethyl Laboratories, Inc., Montgomery, TX) were used as the second antibodies, and standards were a pool of dog serum with known concentrations of canine total IgG, IgG<sub>1</sub> and IgG<sub>2</sub> (Bethyl Laborato-

ries, Inc.). For cat samples, the 2nd antibody was an HRP-conjugated goat anti-cat total IgG antibody, and standards were purified cat IgG (Bethyl Laboratories, Inc.). The sensitivity of these assays was 0.1 to 0.5  $\mu$ g/ml.

### Coagulation and Bethesda assays

The WBCT was performed as described previously [27]. aPTT assay was performed with a Coag-a-mate<sup>R</sup>-XM machine and aPTT reagents from Biomerieux, Inc. (Durham, NC). For quantitation of hFIX activity, 90  $\mu$ l of RV-treated dog plasma was mixed with 10  $\mu$ l of hFIX-deficient human plasma, and the time to clot compared with standards that used 90  $\mu$ l of plasma from untreated hemophilia A dogs, 0 to 10  $\mu$ l of normal human plasma, and 10 to 0  $\mu$ l of hFIX-deficient human plasma. The Bethesda inhibitor assay was performed as described [16]. Briefly, plasma samples were heat-inactivated at 56 °C for 1 h, and 10  $\mu$ l of sample was incubated with 10  $\mu$ l of normal human plasma (George King Biomedical Inc., Overland, KS) at 37 °C for 2 h. Fifty  $\mu$ l of hFIX-deficient human plasma and 30  $\mu$ l of PBS were added, and aPTT assay was performed.

### Evaluation of RV DNA and RNA in cat liver

DNA was isolated from dog or cat livers, and 100 ng was analyzed by real-time PCR using Taqman technology (Applied Biosystems, Rockville, MD) and reagents specific for the WPRE of the RV [28] with normalization to dog [29] or cat [20]  $\beta$ -actin sequences. DNase I-treated RNA was reverse transcribed with reverse WPRE and reverse cat  $\beta$ -actin primers, and real-time PCR was used to determine the RV RNA levels with normalization to the  $\beta$ -actin signal.

### Cytokine secretion assays

Mouse splenocytes were cultured in 24-well plates ( $5 \times 10^6$  cells/well) in medium containing RPMI1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X nonessential amino acids (Cellgro<sup>R</sup>, Mediatech, Inc.), 50 mM  $\beta$ -mercaptoethanol, and 10 mM HEPES. Medium contained 0, 1, or 10  $\mu$ g/ml of hFIX, or 1  $\mu$ g/ml concanavalin A (ConA, Amersham Biosciences, Piscataway, NJ). The concentrations of cytokines were determined by Quantikine<sup>R</sup> ELISA kits (BD Biosciences). IL-4 Elispot assay of mouse lymphocytes used a BD<sup>TM</sup> ELISPOT Mouse IL-4 kit (BD Biosciences). Lymphocytes were cultured for 48 h with medium alone or with medium containing 1 or 10  $\mu$ g/ml of hFIX and spot-forming units were counted.

## Adoptive transfer

Splenocytes were isolated from 3 month-old C3H mice that received  $10^{10}$  TU/kg of hAAT-hFIX-WPRE at 2 to 3 days after birth and were tolerant to hFIX challenge with adjuvant at 2 and 2.75 months, or from 3 month-old naive mice that were never challenged with hFIX. Twenty million cells were injected into 6-week old naive C3H mice via the tail vein. The recipient mice were challenged with IP injection of 30 IU/kg of hFIX with adjuvant at 2 h and 3 weeks after adoptive transfer.

## Statistics

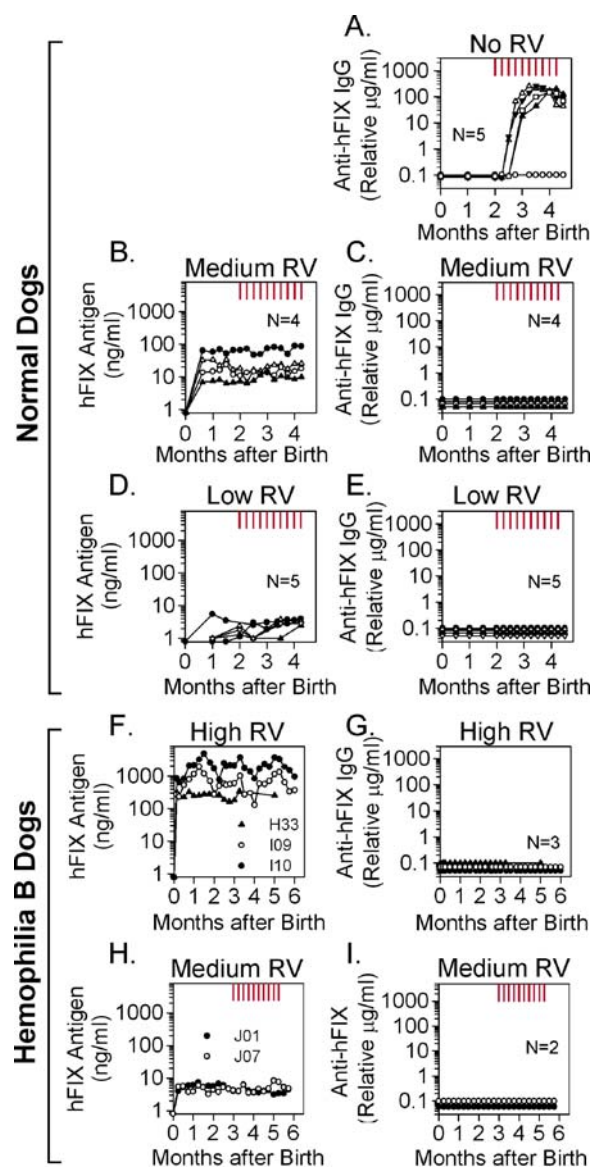
Statistical analyses were performed with SigmaStat software.

## Results

### Neonatal gene transfer induces tolerance to hFIX in normal and hemophilia B dogs

Normal dogs that did not receive RV were challenged IV with hFIX at 30 IU/kg once a week for 10 weeks starting at 2 months of age, as indicated by the red vertical lines in Fig. 1A. Human FIX differs from canine Factor IX at 15% of the amino acids (66 out of 455) and is immunogenic in adult dogs [27]. Indeed, anti-hFIX antibodies developed in 4 of 5 dogs, which averaged  $83 \pm 17$  [standard error of the mean (SEM)]  $\mu\text{g/ml}$  at 1 week after the last challenge (Fig. 1A and Table 1), of which 97.8% was IgG<sub>2</sub> (Th2 response in dogs) [12] and 2.2% was IgG<sub>1</sub> (Th1 response in dogs) (Table 1). The antibodies had inhibitory activity with an average titer of  $11.3 \pm 0.9$  BU/ml (Table 1). These data demonstrate that normal dogs respond to hFIX protein challenge with a Th2 antibody response. Animals that produce antibodies in response to hFIX protein challenge will be referred to hereafter as effectively-immunized.

Normal dogs were transduced as newborns with a medium [ $3 \times 10^8$  transducing units (TU)/kg] or a low ( $4 \times 10^7$  TU/kg) dose of hAAT-hFIX-WPRE, which resulted in hFIX expression of  $28 \pm 12$  ng/ml (0.6% normal) and  $2.1 \pm 0.4$  ng/ml (0.04% normal), respectively, as shown in Fig. 1B, D, and Table 1. Protein challenge with 30 U/kg should result in  $\sim 1500$  ng/ml (30% of normal), which would represent 54- and 714-fold the baseline hFIX levels for the medium and low dose RV groups, respectively. These RV-transduced normal dogs were truly tolerant, as 10 weekly injections of hFIX protein failed to induce anti-hFIX antibodies (Fig. 1C and E) or inhibitors (Table 1). Animals that received neonatal RV and were



**Figure 1** hFIX antigen and anti-hFIX antibody levels in dogs. The data shown in panel A are from normal dogs that did not receive RV (No RV). Normal (panels B to E) or hemophilia B (panels F to I) dogs were injected IV with a high ( $3 \times 10^9$  TU/kg; panels F–G), medium ( $3 \times 10^8$  TU/kg; panels B–C and H–I), or low ( $4 \times 10^7$  TU/kg; panels D–E) dose of RV at 2 to 3 days after birth. Some dogs received IV injection of 30 IU/kg of hFIX protein starting at 2 months of age, which was given once a week for 10 consecutive weeks. The days of protein challenge are indicated as red vertical lines at the tops of panels A, B–E, and H–I. The dogs whose data are shown in panels F and G did not receive protein challenge. Plasma hFIX antigen was determined with an immunoassay that does not cross-react with canine FIX (panels B, D, F, and H), and total anti-hFIX IgG antibodies were measured by ELISA (panels A, C, E, G, and I). In all panels, each line represents values for a single dog. The number of animals (N) in each group is indicated. Normal hFIX levels are 5000 ng/ml. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1** Summary of hFIX antibody formation in different species

Animal model	RV dose (TU/kg)	Peak hFIX antigen (ng/ml)	hFIX protein challenge	Percent of animals with antibodies or inhibitors		Average levels of antibody or inhibitor		Percent Th2 antibody
				Antibody	Inhibitors	Antibody $\mu\text{g/ml}$	BU/ml	
Neonatal normal dogs	None	0 (N=5)	10x	80%	80%	83 $\pm$ 17	11.3 $\pm$ 0.9	97.8 $\pm$ 2.5
	Medium $3 \times 10^8$	28 $\pm$ 12 (N=4)	10x	0%	0%	NA	NA	NP
	Low $4 \times 10^7$	2 $\pm$ 0.4 (N=5)	10x	0%	0%	NA	NA	NP
Neonatal hemophilia B dogs	High $3 \times 10^9$	942 $\pm$ 500 (N=3)	None	0%	0%	NA	NA	NP
	Medium $3 \times 10^8$	5 $\pm$ 0.4 (N=2)	10x	0%	0%	NA	NA	NP
Neonatal normal cats	None	0 (N=2)	10x	100%	100%	55 $\pm$ 10	11.6 $\pm$ 0.4	NT
	High $4 \times 10^9$	118 $\pm$ 29 (N=4)	10x	0%	0%	NA	NA	NT
Neonatal normal C3H mice	<sup>H</sup> None	0 (N=7)	10x	100%	100%	3800 $\pm$ 1400	16 $\pm$ 2	NT
	$1 \times 10^9$	103 $\pm$ 23 (N=8)	10x	12.5%	12.5%	1476	14	81.5 $\pm$ 11.9
	$1 \times 10^8$	2 $\pm$ 1 (N=10)	10x	70%	70%	851 $\pm$ 322	34 $\pm$ 18	92.2 $\pm$ 2.2
Adult normal C3H mice	$1 \times 10^{10}$ (No HGF)	54 $\pm$ 10 (N=4)	None	100%	75%	482 $\pm$ 143	14 $\pm$ 2	88.1 $\pm$ 9.3
	$1 \times 10^{10}$ (+HGF)	1332 $\pm$ 477 (N=4)	None	0%	0%	NA	NA	NP
	$1 \times 10^9$ (+HGF)	47 $\pm$ 16 (N=4)	None	100%	100%	133 $\pm$ 45	11.2 $\pm$ 1.1	85.0 $\pm$ 4.7

<sup>A</sup>hAAT-hFIX-WPRE was injected IV at the indicated TU/kg into some dogs, cats, or mice at 2 to 5 days after birth. Adult transduction in mice was at 6 weeks. <sup>B</sup>For adult gene transfer, mice either did (+HGF) or did not (No HGF) receive HGF prior to RV. <sup>C</sup>The average peak hFIX antigen  $\pm$  SEM and number of animals (N). <sup>D</sup>10x indicates cats or dogs that received 10 doses of 30 IU/kg of hFIX IV once a week, or mice that received 10 doses IP. None indicates no protein challenge. <sup>E</sup>The percent of animals that had total anti-hFIX IgG antibodies  $>20 \mu\text{g/ml}$  or inhibitor titers  $>5 \text{ BU/ml}$  at the completion of the protein challenge, or at 3 months after gene transfer if not challenged. <sup>F</sup>The average anti-hFIX total IgG antibody or inhibitor levels at the times noted above. Only animals with anti-hFIX antibodies above  $20 \mu\text{g/ml}$  or inhibitor titers  $>5 \text{ BU/ml}$  were included in the calculations. NA indicates not applicable as none of the animals had anti-hFIX antibodies. <sup>G</sup>The percent of total anti-hFIX IgG antibodies that were of a Th2 phenotype (IgG<sub>2</sub> in dogs, and IgG<sub>1</sub> in mice) at the time of peak total IgG levels. NP indicates that isotype-specific anti-hFIX antibodies were not present. NT indicates that samples were not tested. <sup>H</sup>C3H mice from the same colony that were challenged in a similar fashion in a previous study and analyzed with the same reagents.

tolerant to hFIX will be referred to hereafter as RV-tolerized.

Hemophilia B dogs were also tested to determine if neonatal gene therapy could correct the bleeding phenotype and induce tolerance to hFIX. Chapel Hill hemophilia B dogs that received a high dose ( $3 \times 10^9$  TU/kg) of RV at 2 or 3 days after birth achieved plasma hFIX levels of  $942 \pm 500 \text{ ng/ml}$  (19% normal), as shown in Fig. 1F. The average WBCT was  $14.7 \pm 0.5 \text{ min}$ , which is close to the normal value of 8 to 13 min, and much improved from the value of  $>60 \text{ min}$  in untreated hemophilia B dogs. The average aPTT was  $19.4 \pm 1.5 \text{ s}$ , which is shorter than the value of  $42.4 \pm 2.6 \text{ s}$  in untreated hemophilia B dogs, but higher than the value of  $9.0 \pm 0.2 \text{ s}$  in normal dogs. hFIX activity was determined by aPTT after dilution of samples and comparison with a standard curve, as detailed in the Methods section. The hFIX functional activity in the last 4 samples that were collected averaged 1.5% of normal for H33, 12.5% for I-09, and 42% for I-10. Since the antigen levels at these times were 5% of normal for H33, 13% of normal for I-09, and 38% of normal for I-10, the percent of protein that was active was 32%, 94%, and 110%, respectively. None

of the high-dose RV-treated dogs had clinically detectable bleeding during 6 months of evaluation, while untreated hemophilia B dogs generally bleed 6 times per year. The hemophilia B dogs that received the high dose of RV did not develop anti-hFIX IgG antibodies (Fig. 1G) or inhibitors (Table 1). These dogs were not challenged with hFIX protein. Their livers contained  $8 \pm 4$  copies of RV DNA per 100 cells at 6 months after transduction (data not shown).

Hemophilia B dogs that received a medium dose ( $3 \times 10^8$  TU/kg) of RV at 2 or 3 days after birth achieved stable expression of hFIX at  $5 \pm 0.4 \text{ ng/ml}$  (0.1% normal), as shown in Fig. 1H. The WBCT was  $20.1 \pm 0.5 \text{ min}$ , and the aPTT was  $34.2 \pm 0.8 \text{ s}$ . The activity of the hFIX was too low in these animals to be quantified. Each of the two medium-dose RV dogs had one minor bleed during 10 months of evaluation. hFIX protein challenge, which should increase hFIX levels to 300-fold the baseline levels, did not induce antibodies (Fig. 1I) or inhibitors (Table 1). In this study, there were no hemophilia B dogs that received challenge with hFIX protein without preceding gene therapy. However, 20 of 20 hemophilia B dogs from the same colony developed

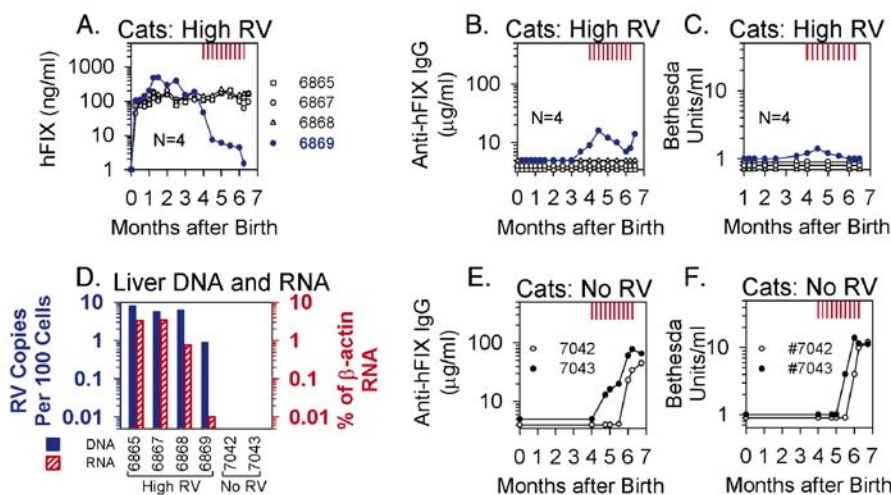
anti-hFIX antibodies after protein challenge in previous studies [27,30]. We conclude that tolerance to hFIX can be achieved after neonatal gene transfer in hemophilia B dogs.

### Immune responses to hFIX with neonatal gene transfer in normal cats

Human Factor IX differs from cat FIX at 82% of the amino acids (82 of 469), and would be expected to be immunogenic in cats. Four newborn normal cats that were injected IV with a high dose ( $4 \times 10^9$  TU/kg) of hAAT-hFIX-WPRE at 5 days after birth achieved  $118 \pm 29$  ng/ml (2% normal) of hFIX in plasma at 1 month, as shown in Fig. 2A, and Table 1. Three cats had stable hFIX levels for 6.5 months and failed to produce anti-hFIX antibodies using ELISA- and coagulation-based anti-hFIX antibody assays even after challenge with hFIX (Fig. 2B and C). In contrast, plasma hFIX levels decreased at 4 months in cat 6869, and were very low at 6.5 months. Cat 6869 had low levels of anti-hFIX antibodies and inhibitors at 4 months, which remained low during hFIX protein challenge. We conclude that none of the cats that received neonatal gene transfer with hAAT-hFIX-

WPRE developed high-titer anti-hFIX antibodies, but 1 of 4 lost expression.

The possibility of a CTL response in cat 6869 was investigated indirectly by analysis of livers for RV DNA and RNA sequences at 6.5 months after RV injection. The three RV-treated cats with stable hFIX expression had  $6.7 \pm 0.7$  copies of RV per 100 cells, as shown in Fig. 2D. For reverse transcriptase real-time PCR of RNA, the difference in the cycle number to reach the threshold ( $C_t$ ) for the WPRE vs.  $\beta$ -actin was used to calculate that RV RNA in liver was  $2.5 \pm 0.8\%$  of  $\beta$ -actin RNA for cats with stable expression. For cat 6869, the liver contained 0.9 copies of RV DNA/100 cells (13% of the value for cats with stable expression), but RV RNA was only 0.01% of  $\beta$ -actin (0.4% of the value for cats with stable expression). We hypothesize that CTLs destroyed cells with high expression in the liver in cat 6869. The discrepancy between DNA and RNA copies is likely due to survival of transduced cells with silent or near-silent integrations, and is very similar to that observed in MPS I cats where a CTL response to IDUA was clearly documented [20]. Liver enzymes, which were elevated in humans with hemophilia B that received gene therapy and



**Figure 2** hFIX antigen, anti-hFIX antibodies, and liver RV nucleic acid levels in cats. Some normal cats were injected IV with a high dose ( $4 \times 10^9$  TU/kg) of hAAT-hFIX-WPRE at 5 days after birth (High RV; panels A to C), while other normal cats did not receive RV (No RV; panels E–F). Cats were challenged with 30 IU/kg of hFIX protein given IV once a week for 10 weeks starting at 4 months of age, as indicated by the vertical red lines at the tops of the graphs. A. hFIX antigen. hFIX antigen was determined with an immunoassay that does not cross-react with feline FIX. Each line represents an individual cat, whose identification number is shown at the right. Values for Cat 6869 are shown with blue closed circles in this and subsequent panels. B and E. Anti-hFIX antibodies. Total anti-hFIX IgG was determined by immunoassay. C and F. Anti-hFIX inhibitors. Anti-hFIX inhibitors were determined by Bethesda assay. D. RV DNA and RNA copies in liver. Livers were collected at 6.5 months of age. The RV DNA copies per 100 cells (filled blue bars) were determined by real-time PCR for the WPRE, with normalization to the  $\beta$ -actin sequence. RV RNA levels (red bars with stripes) were determined by reverse transcriptase real-time PCR for the WPRE with normalization to the  $\beta$ -actin sequence. The ratio of RV RNA to  $\beta$ -actin RNA was determined by comparison of the  $C_T$ . Two non-transduced cats had no signal for RV DNA or RV sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

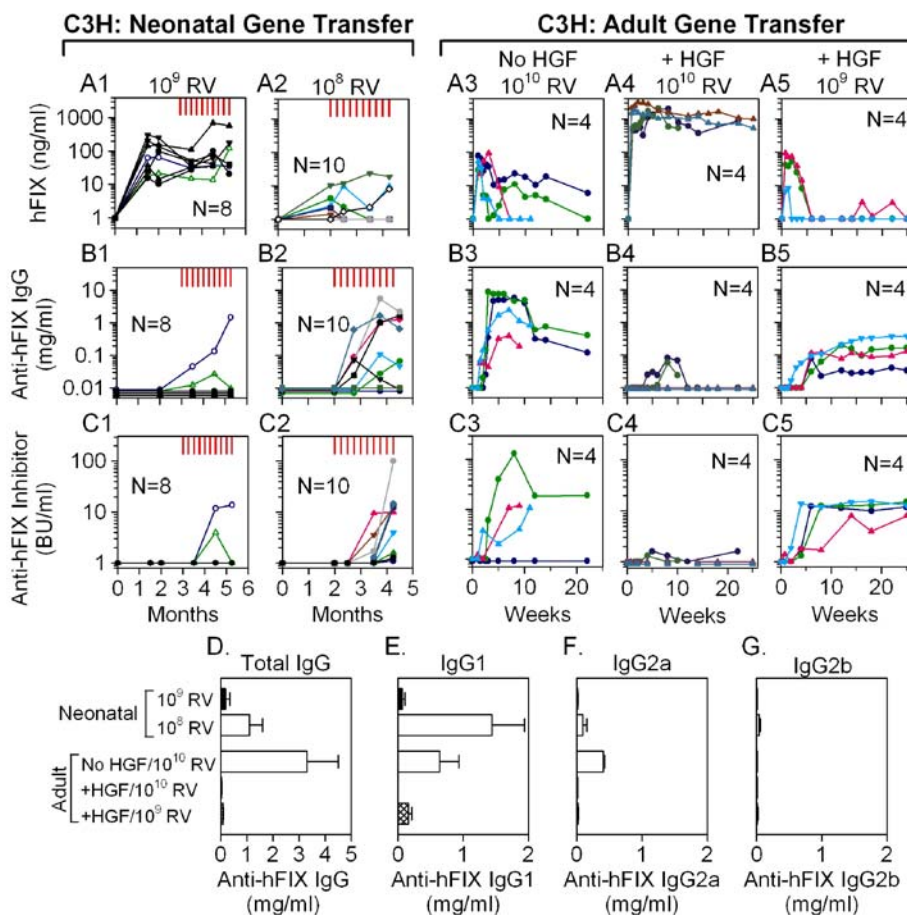
developed a CTL response [31], were not measured in these cats.

To determine if hFIX protein was immunogenic in cats, 2 normal animals were injected IV with 30 U/kg of hFIX once a week for 10 doses. This resulted in  $55 \pm 10 \mu\text{g/ml}$  of total anti-hFIX IgG after 10 weeks, as shown in Fig. 2E. Furthermore, inhibitors were readily detected at  $11.6 \pm 0.4 \text{ BU/ml}$ , as shown in Fig. 2F and Table 1. Thus, the failure to induce high titers of anti-hFIX antibodies in cats after neonatal gene transfer was probably due to

neonatal tolerance rather than an inability of cats to respond to the xenoprotein.

### Immune responses after neonatal or adult gene transfer in C3H mice

Although we previously demonstrated that neonatal gene therapy induced tolerance to hFIX in hemophilia B mice on a C57BL/6 background if plasma levels were  $>14 \text{ ng/ml}$  [16], the mechanism was unclear. Therefore, we performed additional studies in normal C3H



**Figure 3** hFIX antigen and anti-hFIX IgG antibody levels after neonatal or adult gene transfer in C3H mice. For neonatal transfer, C3H mice were injected IV with  $10^9$  TU/kg (panels labeled with a 1) or  $10^8$  TU/kg (panels labeled with a 2) of hAAT-hFIX-WPRE at 2 to 3 days after birth. These mice also received 10 doses of hFIX protein IP at 30 IU/kg/dose once a week for 10 weeks starting at 2 to 3 months after birth, as indicated by the vertical red lines at the tops of the graphs, and were evaluated at the indicated months after gene transfer. Adult gene transfer was performed in 6 week-old C3H mice. The group designated No HGF,  $10^{10}$  RV (panels labeled with a 3) were injected IV with  $10^{10}$  TU/kg of RV without preceding HGF. The group designated +HGF,  $10^{10}$  RV (panels labeled with a 4) received HGF prior to  $10^{10}$  TU/kg of RV. The group designated +HGF,  $10^9$  RV (panels labeled with a 5) received HGF prior to  $10^9$  TU/kg of RV. Mice were evaluated at the indicated weeks after gene transfer. A1–A5. hFIX antigen. hFIX antigen was evaluated at the indicated times after transduction with an immunoassay that does not cross-react with mouse FIX. Each line represents values for an individual mouse, and the same color is used to depict individual mice in panels B1–B5 and panels C1–C5. B1–B5. Anti-hFIX antibodies. Total anti-hFIX IgG was measured by immunoassay. C1–C5. Anti-hFIX inhibitors. Inhibitor assays were performed with the Bethesda assay. D–G. IgG isotypes in mice that received neonatal or adult gene transfer. Anti-hFIX total IgG (panel D), IgG<sub>1</sub> (panel E), IgG<sub>2a</sub> (panel F), and IgG<sub>2b</sub> (panel G) were evaluated by immunoassay. The samples used were those with the peak anti-hFIX total IgG levels, or the last samples collected for RV-transduced mice that did not produce anti-hFIX antibodies. Averages  $\pm$  SEM were plotted and included all animals from each group.

mice, which have a robust antibody response after hFIX protein challenge [16] and do not bleed excessively. Neonatal C3H mice were injected IV with  $10^9$  or  $10^8$  TU/kg of RV at 2 to 3 days after birth. They achieved  $103 \pm 23$  and  $2 \pm 1$  ng/ml of hFIX, respectively, at 2 months after gene transfer, as shown in panels A1 and A2 of Fig. 3, and as summarized in Table 1. None of the mice made anti-hFIX antibodies (panels B1 and B2) or inhibitors (panels C1 and C2) after gene transfer alone. These mice initiated challenge with 30 IU/kg of hFIX given IP once a week for 10 weeks at 2 to 3 months after birth, as indicated by the vertical red lines on the graphs. This dose should increase plasma hFIX to 15- and 789-fold the baseline levels for the  $10^9$  and  $10^8$  TU/kg groups, respectively. Most mice that received  $10^9$  TU/kg of RV were tolerant, as only 1 of 8 had high levels of antibodies ( $>20$   $\mu\text{g/ml}$ ) and high inhibitor activity ( $>5$  BU/ml) at any late time of evaluation. Administration of  $10^8$  TU/kg was less effective, as protein challenge induced high levels of anti-hFIX antibodies and inhibitors in 70% of mice. For comparison, 100% of C3H mice that were challenged with 10 doses of hFIX IP without adjuvant developed high levels of anti-hFIX total IgG antibodies in a previous study [16]. We conclude that high hFIX levels after neonatal gene transfer are probably important for tolerance induction.

Tolerance induction in mice that received neonatal injection of  $10^9$  TU/kg of RV could be due to high expression rather than the immaturity of the newborn immune system, as high plasma hFIX levels can promote tolerance after gene therapy to adult mice [10]. We therefore tested what level of expression would induce tolerance with this RV in 6-week old adult C3H mice. Adult mice that received  $10^{10}$  TU/kg of hAAT-hFIX-WPRE without a stimulus for hepatocyte replication achieved  $54 \pm 10$  ng/ml (1% normal) of hFIX in plasma at 1 week after transduction (Fig. 3, panel A3). However, hFIX expression fell over time, and 100% and 75% developed high levels of anti-hFIX total IgG antibodies and inhibitors, respectively (panels B3, panel C3 and Table 1).

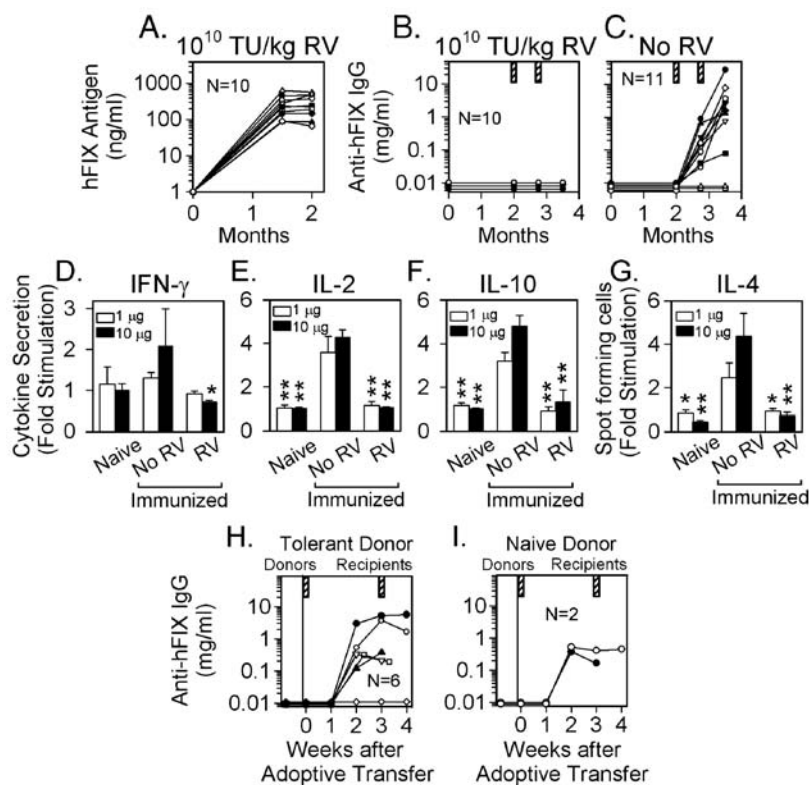
The second group of adult mice received  $10^{10}$  TU/kg of hAAT-hFIX-WPRE during the peak period of HGF-induced hepatocyte replication. They achieved stable expression at  $1332 \pm 477$  ng/ml (27% normal) of hFIX, as shown in Fig. 3, panel A4. Although low levels of anti-hFIX total IgG antibodies were detectable in 2 of 4 mice at 8 weeks after transduction (panel B4), the antibodies fell to undetectable levels by 12 weeks, and had no or little inhibitor activity (panel C4). We conclude that these mice did not develop biologically important levels of antibodies.

It was possible that the HGF used to potentiate transduction affected the immune response, rather than the level of expression. The third group of adult mice therefore received a lower dose ( $10^9$  TU/kg) of RV during the peak period of HGF-induced hepatocyte replication. They achieved  $47 \pm 16$  ng/ml (1% normal) of hFIX in plasma at one week after transduction, which declined to very low levels by 6 weeks (Fig. 3, panel A5). All mice sustained high levels of anti-hFIX total IgG over time (panel B5), and all developed inhibitors (panel C5). Thus, antibodies developed in both groups of mice that achieved  $\sim 50$  ng/ml of hFIX in plasma after adult gene transfer. This suggests that the tolerance that was achieved in newborn mice was due to the immaturity of the immune system rather than the level of expression.

IgG<sub>1</sub> is the major antibody produced in a Th2 response, while IgG<sub>2a</sub> and IgG<sub>2b</sub> are increased in a Th1 response. IgG isotypes were therefore determined for samples with the peak total IgG for each animal, as shown in Fig. 3D-G. For all groups for which isotype assays were positive, IgG<sub>1</sub> was the major anti-hFIX antibody produced (Table 1). This suggests that a Th2 is the most robust response in animals that produced antibodies.

### Mechanism of tolerance after neonatal gene transfer in mice

The mechanism of neonatal tolerance was further evaluated in C3H mice. Newborn C3H mice that were injected IV with  $10^{10}$  TU/kg of hAAT-hFIX-WPRE achieved plasma hFIX levels of  $374 \pm 48$  ng/ml (7% normal) at 2 months (Fig. 4A) and were tolerant to IP injection of 30 IU/kg of hFIX with adjuvant at 2 and 2.75 months of age (indicated by the blue striped bars at the top of Fig. 4B). In contrast, most mice that did not receive RV but were challenged with hFIX made anti-hFIX antibodies (Fig. 4C). Medium from splenocytes from effectively-immunized mice that were incubated with 10  $\mu\text{g/ml}$  of hFIX had IFN $\gamma$ , IL-2, and IL-10 levels that were 2-, 4-, and 4-fold the levels observed for cells that were incubated with medium without hFIX (Fig. 4D-F). IL-2 and IL-10 secretion was also increased when the hFIX concentration in the media was 1  $\mu\text{g/ml}$ . In contrast, secretion of these cytokines was not increased in response to hFIX by splenocytes from naive mice that were never immunized with hFIX (Naive), or by splenocytes from RV-tolerized mice. IL-4 secretion was analyzed with ELISPOT, as protein levels were undetectable in the conditioned media (data not shown). Fig. 4G shows that addition of 1 or 10  $\mu\text{g/ml}$  of hFIX to the medium resulted in  $2.5 \pm 0.7$  or  $4.4 \pm 1.1$  as many IL-4-



**Figure 4** Evaluation of the mechanism of neonatal tolerance in C3H mice. C3H mice were injected IV with  $10^{10}$  TU/kg of RV at 2 or 3 days after birth, or were not injected with RV (No RV). A. hFIX antigen. hFIX antigen was determined by immunoassay of plasma from mice that were transduced as newborns at the indicated months after birth. B–C. Anti-hFIX antibody formation after protein challenge. At 2 and 2.75 months of age, mice received IP injection of 30 IU/kg of hFIX with adjuvant. The times of protein challenge are indicated at the top of the graph with striped bars. Total anti-hFIX IgG was measured by immunoassay. D–F. Cytokine secretion. Splenocytes were isolated at 3 months of age from mice that did not receive RV or protein challenge (Naive,  $N=5$ ), mice that did not receive RV but were challenged with protein (No RV, Immunized,  $N=4$ ), or mice that received both RV and protein challenge (RV, Immunized,  $N=7$ ). Splenocytes were incubated for 48 h with 1 or 10  $\mu$ g/ml of hFIX in the media or with media alone, and medium was tested for IFN $\gamma$  (panel D), IL-2 (panel E), or IL-10 (panel F) levels using immunoassays. Values were calculated as the average ratio in the stimulated (hFIX in media) to the unstimulated (no hFIX in media) samples for individual mice  $\pm$  SEM. Con A resulted in IFN $\gamma$ , IL-2, and IL-10 levels that were  $>10$ -,  $>20$ -, and  $>2$ -fold the levels in medium without hFIX for all preparations. The fold stimulation in the naive and RV-treated mice was compared with the values at the same hFIX concentration for effectively-immunized mice with ANOVA with Tukey post-hoc analysis. \* indicates a  $p$  value of 0.01 to 0.05, and \*\* indicates a  $p$  value  $<0.01$ . G. IL-4 ELISPOT. ELISPOT was used to determine the number of IL-4-secreting cells, and the fold stimulation with hFIX in the medium was determined. ConA increased the number of IL-4-secreting cells by  $>10$ -fold for each sample. H. Adoptive transfer of tolerant lymphocytes. Splenocytes were obtained from RV-tolerized C3H donors that received neonatal  $10^{10}$  TU/kg of RV and did not produce antibodies to hFIX after challenge (see panel B).  $2 \times 10^7$  splenocytes were injected IV into congenic C3H mice, and recipients were immunized with hFIX in adjuvant at 30 IU/kg/dose at 2 h and at 3 weeks after the injection of splenocytes. The days of hFIX protein challenge are shown as blue striped bars at the tops of the panels. Plasma was tested for anti-hFIX IgG at the indicated time after adoptive transfer. I. Adoptive transfer of naive lymphocytes. Adoptive transfer was performed as in panel G using lymphocytes from naive C3H mice that did not receive RV or immunization with hFIX.

secreting cells, respectively, as when cells were plated without hFIX. hFIX did not increase the number of IL-4-secreting cells for naive or RV-tolerized mice.

Adoptive transfer experiments were performed to determine if splenocytes from tolerant mice could suppress an immune response in naive recipients. Naive C3H mice were injected IV with  $2 \times 10^7$  splenocytes from C3H mice that were tolerized by neonatal gene transfer (see Fig. 4B),

and the recipients were challenged IP with 30 IU/kg of hFIX with adjuvant 2 h and 3 weeks later. Four of five recipients developed a robust antibody response (Fig. 4H) that was similar in magnitude and time course to that observed in mice that did not receive adoptive transfer (not shown), or received adoptive transfer of splenocytes from naive mice (Fig. 4I). We conclude that lymphocytes from tolerant donors did not confer tolerance upon the recipients.

## Discussion

### Low levels of hFIX expression after neonatal gene transfer induce tolerance in normal and hemophilia B dogs

These data demonstrate that neonatal gene transfer with a high ( $3 \times 10^9$  TU/kg), medium ( $3 \times 10^8$  TU/kg), or low ( $4 \times 10^7$  TU/kg) dose of hAAT-hFIX-WPRE does not induce antibodies or inhibitors to hFIX in normal or hemophilia B dogs, and resulted in tolerance to challenge with hFIX. It was surprising that dogs with plasma hFIX levels as low as 2 ng/ml (0.04% normal or  $4 \times 10^{-11}$  M) were tolerant to hFIX protein infusion (Fig. 1), as this level did not induce tolerance in hemophilia A:C57BL/6 mice in a previous study [16], or in normal C3H mice in this study (Fig. 3). It is unclear why the level of expression needed to induce tolerance differs between mice and dogs.

### Cats do not develop antibodies after neonatal gene therapy but can develop a CTL response

None of 4 cats that received high-dose RV and achieved peak plasma hFIX levels of  $118 \pm 29$  ng/ml (2% normal) developed high levels of antibodies either before or after protein challenge (Fig. 2). However, 1 cat lost expression at 4 months, which was associated with RV RNA levels that were only 0.4% of that observed in cats with stable expression. The loss of expression in this cat was probably not due to promoter silencing, as each liver has many cells with different integration sites. Since the CTL response to cIDUA in 100% of MPS I cats that received neonatal gene therapy was also associated with a marked decline in RV RNA levels [20], it is very likely that a CTL response destroyed RV-transduced cells in this cat. It remains unclear as to what the frequency of a CTL response would be if a species-specific transgene had been used in cats with hemophilia B, or if animals that were more outbred had been used. Future studies will directly test if neonatal gene transfer induces a CTL response to hFIX in cats, and if this can be blocked with CTLA4-Ig, which blocks the CTL response to cIDUA in MPS I cats after neonatal gene therapy [20]. Mice can destroy cells that are loaded with hFIX peptides *in vitro* [32], although they do not appear to eradicate hFIX-expressing cells in the liver or muscle for reasons that are unclear. In addition, CTL responses to viral capsid proteins probably caused the loss of expression in human patients

after adult gene therapy [31]. Induction of a CTL response after neonatal gene therapy may be potentiated by transduction of antigen-presenting cells, as spleen cells of an unknown phenotype are transduced in mice [33], dogs [34], and cats (MEH and KPP, unpublished data).

### The mechanism of tolerance after neonatal gene transfer may involve clonal deletion

Splenocytes from effectively-immunized C3H mice increase their secretion of IFN $\gamma$ , IL-2, IL-4, and IL-10 in response to hFIX (Fig. 4). In contrast, splenocytes from RV-tolerized C3H mice did not increase cytokine secretion if hFIX was added to the medium. IFN $\gamma$  and IL-2 are classically associated with CTL responses or the production of Th1-type antibodies (IgG $_1$  in dogs, and IgG $_{2a}$  and IgG $_{2b}$  in mice), while IL-4 is a critical cytokine for Th2-type antibodies (IgG $_2$  in dogs and IgG $_1$  in mice) [35,36]. In this study, the secretion of IFN $\gamma$  and IL-2 in lymphocytes from effectively-immunized mice is consistent with the modest elevations in the Th1-type antibodies, while secretion of IL-4 is consistent with the marked elevation of Th2-type antibodies. Our results are similar to the results of Wang et al. [37], who reported secretion of IFN $\gamma$  and IL-2 in response to hFIX in effectively-immunized mice after administration of an AAV vector to adults and a predominant Th2-type anti-hFIX antibody profile.

Although IL-10 was originally described as a cytokine produced by Th2 cells, it is also produced by T regulatory cells (T $_{regs}$ ), which can inhibit immune responses [38]. The increase in IL-10 secretion by effectively-immunized mouse splenocytes in response to hFIX is consistent with the Th2 antibody profile. The failure to increase IL-10 secretion in RV-tolerized C3H mice could indicate the lack of a robust Th2 response, or an absence of T $_{regs}$ . Adoptive transfer of splenocytes from RV-tolerized C3H mice failed to confer tolerance to hFIX, which suggests that the mechanism of tolerance does not involve suppression. However, it remains possible that the time of immunization was not optimal, or that adoptive transfer of T $_{reg}$  subsets such as CD4 $^+$ /CD25 $^+$  cells [39] would be more effective. Taken together, these data suggest that clonal deletion may be the main mechanism of tolerance after neonatal gene transfer. This is consistent with studies on the mechanism of neonatal tolerance in other settings [40–42], although suppression can also play a role [22]. It has also been reported that liver gene transfer in adults results in clonal deletion [43],

although suppressor cells probably also played a role [44].

It remains unclear why the immune system usually induces tolerance after neonatal gene transfer. Newborns from humans and/or mice have reduced secretion of cytokines by lymphocytes [45,46], have fewer dendritic cells [47], reduced responses of dendritic cell precursors to cytokines [48], and low levels of the co-stimulatory molecule CD40 ligand [49].

### Implications for gene therapy for hemophilia

The goal of this study was to determine if immune responses would occur after neonatal gene transfer in animals to guide future attempts at gene therapy in humans with hemophilia. Inhibitor development after gene therapy is a feared complication, as it would make a patient's disease difficult to treat. The demonstration that dogs, cats, and mice failed to develop significant levels of inhibitors to hFIX after neonatal gene therapy is very reassuring. In addition, inhibitor formation is infrequent (<5%) in patients with hemophilia B, most of which have missense mutations [2]. These findings reduce the chance that humans would develop inhibitors after neonatal gene therapy for hemophilia B, and suggest that initial trials should not require concurrent immunosuppression for the sole purpose of blocking inhibitor formation. However, 1 of 4 cats appeared to lose expression due to a CTL response to hFIX after neonatal gene therapy, although this did not occur in any of over 70 mice, or in any of 19 dogs from this and our previous [16] study. This frequency would likely be even lower in patients with missense mutations, with only 1 new epitope with the potential to interact with the immune system. Furthermore, a CTL response would not affect the ability to resume standard therapy, which is protein administration. Thus, although CTL responses remain a concern, the low frequency observed to date suggests that initial trials in humans should not require inhibition of CTL responses with immunosuppression. However, it remains uncertain as to what the response to the more immunogenic Factor VIII protein will be, which is the focus of ongoing studies. It is possible that initiation of protein therapy at birth could induce long-standing tolerance, as was achieved in 58% of hemophilia B dogs with prolonged subcutaneous administration of hFIX starting at birth [27]. Future studies should define the optimal dose, frequency, and duration of neonatal protein administration to induce tolerance.

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