Nonclinical Pharmacology/Toxicology Study of AAV8.TBG.mLDLR and AAV8.TBG.hLDLR in a Mouse Model of Homozygous Familial Hypercholesterolemia

Jenny A. Greig,[1,†] Maria P. Limberis,[2,†] Peter Bell,[1] Shu-Jen Chen,[1] Roberto Calcedo,[1] Daniel J. Rader,[3,4] and James M. Wilson[1,*]

[1]Gene Therapy Program, Department of Medicine; [2]Department of Pathology and Laboratory Medicine; [3]Division of Translational Medicine and Human Genetics, Department of Medicine; [4]Department of Genetics; University of Pennsylvania, Philadelphia, Pennsylvania.
[†]These authors contributed equally to this work.

The homozygous form of familial hypercholesterolemia (HoFH) is an excellent model for developing *in vivo* gene therapy in humans. The success of orthotropic liver transplantation in correcting the metabolic abnormalities in HoFH suggests that the correction of low-density lipoprotein receptor (LDLR) expression in hepatocytes via gene therapy should be sufficient for therapeutic efficacy. Vectors based on adeno-associated virus serotype 8 (AAV8) have been previously developed for liver-targeted gene therapy of a number of genetic diseases, including HoFH. In preparation for initiating a Phase 1 clinical trial of AAV8-mediated LDLR gene therapy for HoFH, a combined pharmacology/toxicology study was conducted in a mouse model of HoFH. No dose-limiting toxicities were found at or below 6.0×10^{13} GC/kg. Therefore, the maximally tolerated dose is greater than the highest dose that was tested. Mild and transient liver pathology was noted at the highest dose. Therefore, the no effect dose was greater than or equal to the middle dose of 7.5×10^{12} GC/kg. The minimally effective dose was determined to be $\leq7.5\times10^{11}$ GC/kg, based on stable reductions in cholesterol that were considered to be clinically significant. This translates to a therapeutic window of ≥80-fold for the treatment of HoFH.

Keywords: AAV, gene therapy, familial hypercholesterolemia, HoFH, LDLR, low density lipoprotein receptor

INTRODUCTION

THE LIVER, WHICH PLAYS a central role in the systemic homeostasis of many metabolic pathways, has emerged as a favored target for *in vivo* gene therapy. Gene correction of hepatocytes alone may be therapeutic for the treatment of genetic disorders, and systemically delivered vectors readily target hepatocytes due to fenestrae in the capillary walls of the liver. Vectors based on adeno-associated viruses (AAV) are attractive vehicles for gene therapy, and a new family of endogenous primate AAVs shows high capacity for efficient and safe *in vivo* gene delivery.[1,2] One of the more hepatotropic vectors, based on AAV serotype 8 (AAV8), has shown success in a number of preclinical and clinical models, including patients with hemophilia B.[3–6] As a result, the development of liver-targeted, AAV8-mediated gene therapy has been pursued for the homozygous form of familial hypercholesterolemia (HoFH), which is caused by a severe deficiency of low density lipoprotein receptor (LDLR).[7,8]

The most commonly used mouse model for HoFH studies has been the $Ldlr^{-/-}$ mouse. Although LDLR deficiency leads to a dramatic phenotype of pronounced hypercholesterolemia and myocardial infarction in humans, deletion of LDLR in the mouse is not associated with hypercholesterolemia when fed a normal chow diet.[9–12] Instead, hypercholesterolemia in the $Ldlr^{-/-}$ mouse must be induced through a high-fat diet, which does not recapitulate the lipoprotein profile and pathogenesis of atherosclerosis seen in humans. A variation in the metabolism of apolipoprotein B (ApoB) may explain the phenotypic difference observed between mouse and man.[13] Humans synthesize the full-length version of ApoB, called ApoB100, which is incorporated

*Correspondence: Dr. James M. Wilson, Gene Therapy Program, Perelman School of Medicine, University of Pennsylvania, 125 South 31st Street, Philadelphia, PA 19104. E-mail: wilsonjm@upenn.edu

DOI: 10.1089/humc.2017.007

into LDL particles to render them as high affinity ligands for LDLR. Conversely, *ApoB* mRNA editing catalytic polypeptide-1 (Apobec1) is highly expressed in mouse liver, resulting in the production of a truncated form of ApoB (ApoB48) that does not bind to LDLR. ApoB48 is cleared more rapidly from the blood through processes that are independent of LDLR, possibly explaining why $Ldlr^{-/-}$ mice do not develop significant hypercholesterolemia or atherosclerosis on a normal rodent chow diet.[14]

Recognizing that ApoB48 activity is a major difference between mice and humans in terms of lipoprotein metabolism, a germ line ablation was introduced into the *Apobec1* gene in the mouse. A phenotype very similar to human HoFH was created when the Apobec1-ablation was bred into an $Ldlr^{-/-}$ strain of mice.[15] These double Ldlr/Apobec-1 knockout (DKO) mice, which are deficient in both LDLR and Apobec1, develop severe hypercholesterolemia due to elevations in LDL when fed a normal chow diet. This is associated with atherosclerosis that is similar in distribution and histology to what is seen in HoFH patients.

AAV8 vectors expressing murine and human versions of LDLR have been previously developed and tested in the DKO mouse model, which resulted in complete correction of hypercholesterolemia and reversal of pre-existing atherosclerosis.[16,17] The present study describes a combined pharmacology/toxicology investigation of the clinical candidate vector, AAV8.TBG.hLDLR, in DKO mice to support the initiation of a Phase 1 clinical trial in HoFH patients.

MATERIALS AND METHODS

Vector preparation

AAV vectors expressing either mouse LDLR (mLDLR) or human LDLR (hLDLR) from the thyroxine binding globulin (TBG) promoter were produced by the Penn Vector Core at the University of Pennsylvania, as previously described.[18] The vector was stored frozen at –60°C to –80°C. The final vector products were diluted in sterile phosphate-buffered saline (PBS; Mediatech, Inc., Corning Life Sciences, Corning, NY) and kept on wet ice or at 4°C until injection. A certificate of analysis that verifies quality and purity is provided in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/humc).

Mouse model of HoFH

A total of 280 (140 male, 140 female) homozygous DKO mice were used in this study. The original mice were obtained from the laboratory of Dr. Daniel J. Rader and were bred and acclimated in a University of Pennsylvania facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and assured by the Public Health Service. All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. All DKO mice were genotyped prior to enrollment in the study. Mice were between 6 and 22 weeks of age and weighed between 14.6 and 37.5 g at the initiation of the study. Each animal was identified with a unique identification number on an ear tag. Following determination of the weight, age, and sex of the animals to be included in the study, each treatment group included (a) an equal number of males and females, (b) an equal representation of the various ages, and (c) an equal representation of mouse weights.

Up to five mice per cage were housed in solid-bottom, polypropylene micro-isolator cages with corn cob bedding. All cage sizes and housing conditions were in compliance with the Guide for the Care and Use of Laboratory Animals. Cages, water bottles, feed, and bedding substrates were autoclaved into the barrier facility. An automatically controlled 12 h light/dark cycle was maintained. Animals were fed Purina Lab Diet Rodent 5010, and local water that had been processed by passage through a Getinge water purifier was available to the mice *ad libitum* via an individually placed water bottle in each housing cage (chlorination level of two to four parts per million).

Mice were administered with vector intravenously (i.v.) via the tail vein in a volume of 100 μL using a 27 gauge 1/2 inch needle and 1 mL syringe.

Justification of dosing and time points

The doses used for the vector that expresses the murine version of LDLR were 7.5×10^{11} GC/kg, 7.5×10^{12} GC/kg, and 6.0×10^{13} GC/kg of AAV8.TBG.mLDLR, as determined by quantitative polymerase chain reaction (qPCR) titer. The lowest dose of 7.5×10^{11} GC/kg represented the initial dose of the proposed clinical trial (Clinicaltrials.gov, NCT02651675), and a measurable decrease in total serum cholesterol was predicted at this dose. The middle dose of 7.5×10^{12} GC/kg represented the highest proposed dose of the clinical trial. In the preclinical efficacy studies, >95% reduction in total serum cholesterol was seen in AAV8.TBG.mLDLR-administered DKO mice at this dose. The highest dose of this preclinical study was 6.0×10^{13} GC/kg, which is approximately eightfold greater than the highest dose of the proposed clinical trial. No increases in

serum levels of transaminases were observed at any of these doses in the efficacy studies.

One additional vector cohort included mice injected i.v. with 6.0×10^{13} GC/kg of the clinical candidate vector, AAV8.TBG.hLDLR. This dose is approximately eightfold greater than the highest dose intended for the proposed clinical trial. This group provides important information on the margin of safety between the highest anticipated dose in the clinical trial and the potential maximal tolerated dose. The indicated time points (day 3, day 14, day 90, and day 180 post vector administration) were selected to capture the expression profile of the vector. For a summary of the study design, see Table 1.

Analyses of blood samples

Blood was collected retro-orbitally prior to study initiation or by cardiac puncture at necropsy. Blood collected at necropsy was submitted to Antech Diagnostics (Irvine, CA) for clinical chemistries. Serum collected at baseline, day 3, and day 14 post vector administration with 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR was evaluated for the presence of inflammatory cytokines by multiplex enzyme-linked immunosorbent assay (ELISA; Luminex) with pre-injection serum as a baseline. Cytokine and chemokine elevations in serum were measured using a 32 analyte assay (see Supplementary Table S2 for a list of cytokines/chemokines analyzed). Briefly, the analytes were evaluated using a Mouse Milliplex kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Plates were read on a Luminex 200 instrument (Luminex Corporation, Austin, TX) at the Human Immunology Core at the University of Pennsylvania, and the data were analyzed using Bioplex manager (Bio-Rad, Hercules, CA). The lower limit of detection for this assay was 12.8 pg/mL for interleukin-13 and 3.2 pg/mL for all other analytes.

Neutralizing antibody (NAb) titers were determined on serum samples collected on day 28 post vector administration from mice administered with 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR, as previously described.[19]

Necropsy and analysis of tissues for pathology

Mice were euthanized at the scheduled necropsy time points. The list of tissues collected is provided in Supplementary Table S3. At the time of necropsy, tissues were collected for RNA expression in a manner that reduced the potential for cross-contamination and were snap frozen. RNA levels were quantified using qPCR, as previously described.[20,21]

All tissues collected for histopathology were fixed using 10% neutral-buffered formalin, paraffin embedded, sectioned, and stained for histopathology assessments using hematoxylin and eosin stain. Slides were examined microscopically by a board-certified veterinary pathologist at Charles River Laboratories, Pathology Associates (Wilmington, MA). Findings in the lung were peer re-

Table 1. *Summary of study design*

Cohort	*Vector/vehicle control*	*Dose (GC/kg)*	*Necropsy time point*	*Number of males*	*Number of females*
1	PBS	—	Day 3	7	7
	AAV8.TBG.mLDLR	7.5×10^{11}	Day 3	7	7
	AAV8.TBG.mLDLR	7.5×10^{12}	Day 3	7	7
	AAV8.TBG.mLDLR	6.0×10^{13}	Day 3	7	7
2	PBS	—	Day 14	7	7
	AAV8.TBG.mLDLR	7.5×10^{11}	Day 14	7	7
	AAV8.TBG.mLDLR	7.5×10^{12}	Day 14	7	7
	AAV8.TBG.mLDLR	6.0×10^{13}	Day 14	7	7
3	PBS	—	Day 90	7	7
	AAV8.TBG.mLDLR	7.5×10^{11}	Day 90	7	7
	AAV8.TBG.mLDLR	7.5×10^{12}	Day 90	7	7
	AAV8.TBG.mLDLR	6.0×10^{13}	Day 90	7	7
4	PBS	—	Day 180	7	7
	AAV8.TBG.mLDLR	7.5×10^{11}	Day 180	7	7
	AAV8.TBG.mLDLR	7.5×10^{12}	Day 180	7	7
	AAV8.TBG.mLDLR	6.0×10^{13}	Day 180	7	7
5	AAV8.TBG.hLDLR	6.0×10^{13}	Day 3	7	7
	AAV8.TBG.hLDLR	6.0×10^{13}	Day 14	7	7
	AAV8.TBG.hLDLR	6.0×10^{13}	Day 90	7	7
	AAV8.TBG.hLDLR	6.0×10^{13}	Day 180	7	7

PBS, phosphate-buffered saline.

viewed by a second board-certified pathologist at the University of Pennsylvania.

T-cell responses to AAV8 capsid and h*LDLR* transgene

Splenocytes were harvested on day 14 post vector administration from animals that received 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR. T-cell responses to AAV8 and h*LDLR* were analyzed by interferon gamma (IFN-γ) enzyme-linked immunospot assay (ELISPOT), as previously described,[22] using peptide libraries specific for the AAV8 capsid and h*LDLR* transgene, where positive response criteria are >55 spot forming units (SFU)/10^6 cells and three times the medium negative control value (no stimulation).

Statistical analyses

A formal evaluation for statistical significance was performed on all clinical pathology values by a biostatistician. Comparisons between parameters for each group were performed versus the control group for assessment on days 3, 14, 90, and 180 post vector administration. The comparisons were stratified by sex and were examined descriptively using summary statistics and visual boxplots. Inferential comparisons were carried out using general linear modeling within SAS v9.4 (SAS Institute, Cary, NC) using Proc Genmod. Statistical significance was taken at the 0.05 level without regard to multiple comparisons or power. Additional analyses were performed in Prism (GraphPad Software, San Diego, CA). An unpaired Student's *t*-test was used for comparison of RNA levels, and comparisons between multiple groups for evaluation of cytokine levels were performed using one-way analysis of variance (ANOVA; Tukey's multiple comparison post-test). All values are expressed as mean ± standard error of the mean (SEM).

RESULTS

Study rationale and design

The DKO mouse model simulates the metabolic and clinical aspects of HoFH as they develop severe hypercholesterolemia and extensive atherosclerosis on a chow diet.[15] Additionally, this mouse line has been bred onto a C57BL/6 background, enabling analysis for antigen-specific T cells restricted by the H-2b haplotype.

This study was designed to test the clinical candidate, AAV8.TBG.hLDLR, at a dose of 6.0×10^{13} GC/kg, which is approximately eightfold higher than the highest dose that would be administered to subjects with HoFH in the proposed clinical trial. A version of the vector that expresses the murine version of LDLR was tested at this high dose, as well as at two lower doses, to provide an assessment of the effect of dose on toxicity parameters and cholesterol reduction. The dose–response experiment to establish the minimally effective dose was performed with the vector expressing murine LDLR, since it should better reflect the efficacy that would be observed in humans using the human LDLR vector. The data show that the hLDLR vector underestimates efficacy in the DKO mouse, and it may also exaggerate toxicity,[16,17] especially in regards to T-cell responses to the transgene product (*i.e.*, the mouse may mount a greater T-cell response to human LDLR than to mouse LDLR).

A summary of the study design is included in Table 1. On study day 0, mice were administered with 7.5×10^{11} GC/kg, 7.5×10^{12} GC/kg, or 6.0×10^{13} GC/kg of AAV8.TBG.mLDLR, 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR, or vehicle control (PBS). Vector and vehicle control were both administered as a bolus 100 μL i.v. injection. Four necropsy time points were chosen for the study: day 3, day 14, day 90, and day 180 post vector administration. Day 3 was chosen as an early time point to examine the presence of innate inflammatory responses against the vector. Maximal transgene expression occurred on day 14. Therefore, this time point was important for examining the activation of adaptive immune responses, such as cytotoxic T lymphocytes (CTLs), against both the capsid and transgene. Day 90 represented an intermediate time point that enabled us to examine the presence of toxicity after maximum transgene expression has stabilized in the absence of primary CTL responses, and day 180 represented a long-term time point.

Clinical findings

During the course of the study, two mice were euthanized outside of the designated necropsy time points. Upon the request of the clinical veterinarian, the first of these two mice (a female mouse injected with PBS and scheduled for necropsy on day 90 post vector administration) was euthanized on day 5 of the study due to an ulcerated mass on the lower jaw and across the chest. The pathologist reported no microscopic findings that would explain the need to euthanize this animal. The second animal (a male mouse injected with 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR and scheduled for necropsy on day 90 post vector administration) was euthanized on day 1 of the study due to signs of distress seen at daily viability checks. The pathologist found marked neutrophilic meningoenceph-

alitis with bacteria, which was interpreted as the cause of death.

Following administration of vector or vehicle control, animals were monitored daily for general observations. With the exception of the mice that were euthanized prior to their necropsy time points and the occasional requirement of ear retagging, there were little to no observations reported.

Clinical pathology

The blood chemistry and cell profiles of the animals were analyzed at the time of necropsy by the contract facility, Antech Diagnostics. Thirty different parameters of the blood chemistry results were evaluated for statistical significance ($p < 0.05$) compared with mice administered with the vehicle control (100 μL of PBS) at each necropsy time point. None of these measures revealed dose-dependent and consistent abnormalities in the vector administered groups, except for a predicted decline in cholesterol.

Due to concerns over liver toxicity, the results of liver function tests (LFTs) have been included in Fig. 1. Previous studies in DKO and LAHB mice, both of which are hypercholesterolemic in the absence of a high-fat diet and do not have steatosis, did not show toxicity following either m*LDLR*- or h*LDLR* AAV8-mediated gene transfer.[16,17] Here, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were monitored at the time of necropsy as part of the clinical chemistry testing performed by Antech Diagnostics (Fig. 1A and 1B, respectively). Abnormalities in LFTs were restricted to elevations in AST and ALT, which were less than or substantially lower than four times the upper limit of normal (ULN) in all cases. Elevations in AST were limited to a few male mice necropsied on day 90 post vector administration with each of the three doses of the mLDLR vector. The only female mice with elevations of AST were from day 90 post vector administration with the mid-dose mLDLR vector. Elevations of ALT gave a similar pattern, with increases observed on day 90 in samples from the mid- and high-dose cohorts of the mLDLR vector, and a sporadic elevation from day 3 female mice administered with the hLDLR vector. The findings with the high-dose hLDLR

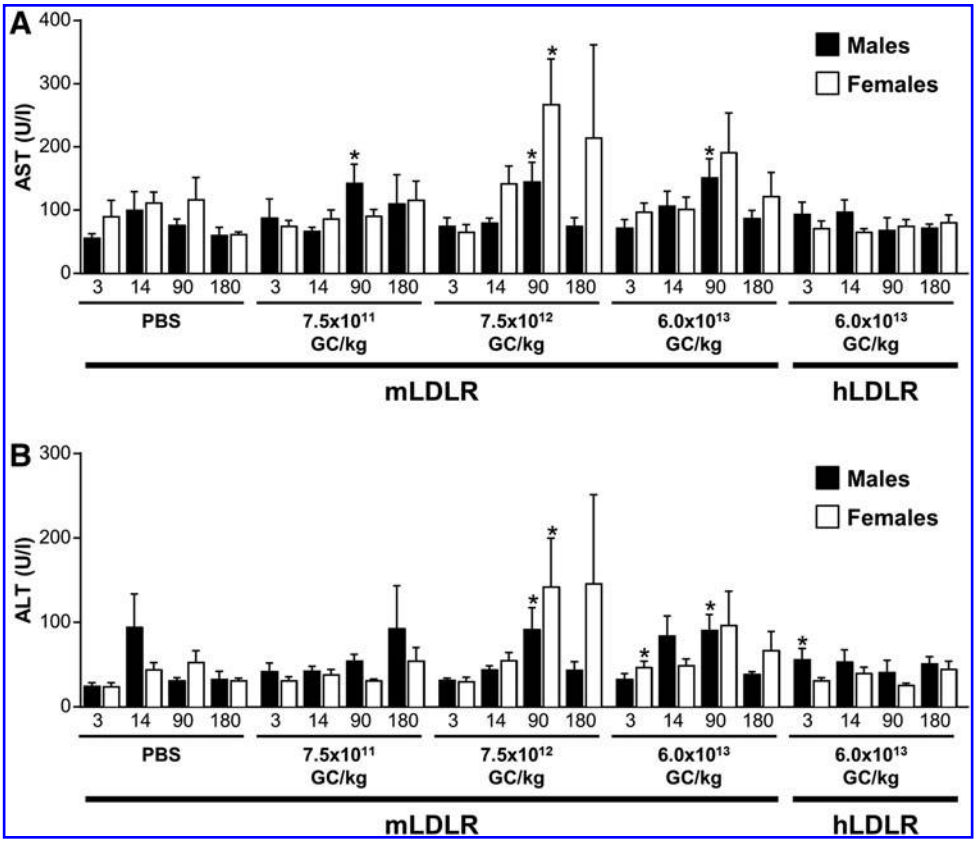

Figure 1. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in DKO mice injected with vector or vehicle control. DKO mice were injected intravenously (i.v.) with 7.5×10^{11} GC/kg, 7.5×10^{12} GC/kg, or 6.0×10^{13}GC/kg of AAV8.TBG.mLDLR, 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR, or vehicle control (100 μL of phosphate-buffered saline [PBS]). (**A**) AST and (**B**) ALT levels were measured in serum samples taken at the time of necropsy by Antech Diagnostics. Values expressed as mean ± standard error of the mean (SEM). Statistically significant elevations in AST and ALT levels relative to controls at the same necropsy time point are indicated (*$p < 0.05$; $n = 7$/sex/group).

vector were minimal, with only a few male animals demonstrating an elevation less than twice the ULN in ALT on day 3. All other groups fell within the normal limits. Notably, other parameters of liver toxicity, such as bilirubin, were normal for all groups. The mechanism(s) responsible for low-level transient elevations in transaminases on day 90 are unclear. However, they seem to be related to vector, since they were not observed in PBS-injected animals. It is unlikely that transgene product- or capsid-specific immune responses were involved, since the abnormalities were observed with what should be the less immunogenic transgene product (*i.e.*, murine LDLR vs. human LDLR).

Effect of vector on serum cholesterol levels

The serum cholesterol levels of all animals were measured at the time of necropsy by the contract facility, Antech Diagnostics. Cholesterol levels in DKO mice injected with the vehicle control were similar across all necropsy time points, with no variation in levels between males and females (Fig. 2). As expected, a rapid and significant reduction of cholesterol was observed on day 3, which persisted through days 14, 90, and 180 post vector administration in both male and female mice administered at all doses of the mLDLR vector (7.5×10^{11} GC/kg, 7.5×10^{12} GC/kg, and 6.0×10^{13} GC/kg), as well as 6.0×10^{13} GC/kg of the hLDLR vector ($p<0.05$).

There was an apparent sex difference seen in the DKO mice following i.v. administration of the low dose (7.5×10^{11} GC/kg) mLDLR vector, which resulted in an average reduction in serum cholesterol of 62% in male DKO mice and 36% in female DKO mice across the four time points tested. There was no sex affect for the mid and high doses of the mouse LDLR vector or for the high-dose human LDLR vector. Administration of 7.5×10^{12} GC/kg of mouse LDLR vector resulted in a >80% reduction in serum cholesterol (Fig. 2). This dose represents the highest proposed dose of the clinical trial. Increasing the dose above 7.5×10^{12} GC/kg of either the mouse or human LDLR vector did not provide a further decrease in serum cholesterol, which was close to normal levels (Fig. 2). In general, the reduction in serum cholesterol seen by day 14 was the same as that seen on day 180 post vector administration. All groups of animals administered with the mLDLR vector and the one high dose of the hLDLR vector showed statistically significant declines in serum cholesterol ($p<0.05$) that persisted for the duration of the study, which was 180 days.

Cytokine analysis

The presence of inflammatory cytokines/chemokines in serum samples from four male and four female mice administered with the high dose (6.0×10^{13} GC/kg) of AAV8.TBG.hLDLR was measured by multiplex ELISA assay (Luminex) on serum samples from day 3 and day 14 post vector administration using pre-vector injection serum as a baseline.

Baseline levels of granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-1β, IL-2, IL-4, IL-3, IL-10, LIF, IL-17, MIP-1β, vascular endothelial growth factor, and tumor necrosis factor alpha were below the range of detection or did not meet the inclusion criteria (results available for two or more animals out of the four tested). Therefore, no further analysis could be performed for these parameters. IL-6, IL-9, KC, MCP-1, MIP-1α, and MIP-2 levels are presented in Fig. 3. Levels were not determined for baseline samples collected

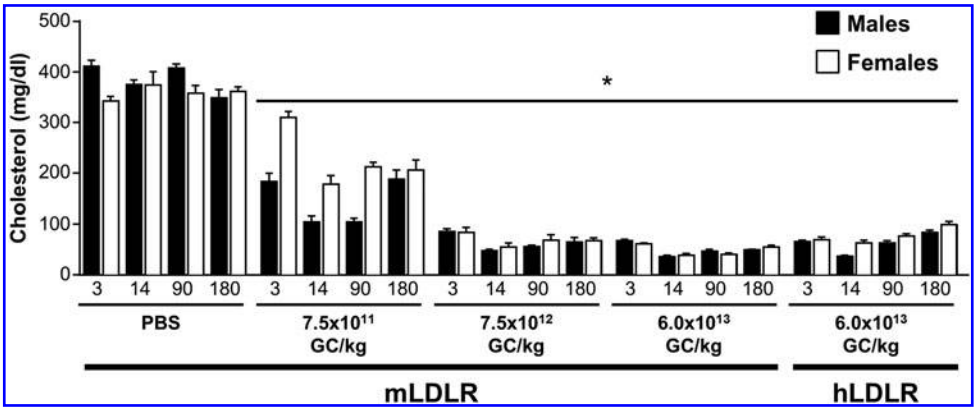

Figure 2. Cholesterol levels in DKO mice injected with vector or vehicle control. DKO mice were injected i.v. with 7.5×10^{11} GC/kg, 7.5×10^{12} GC/kg, or 6.0×10^{13} GC/kg of AAV8.TBG.mLDLR, 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR, or vehicle control (100 μL of PBS). Cholesterol levels were measured in serum samples taken at the time of necropsy by Antech Diagnostics. Values expressed as mean ± SEM. Each group demonstrated a statistically significant reduction in serum cholesterol relative to controls at the same necropsy time point (*$p<0.05$; $n=7$/sex/group).

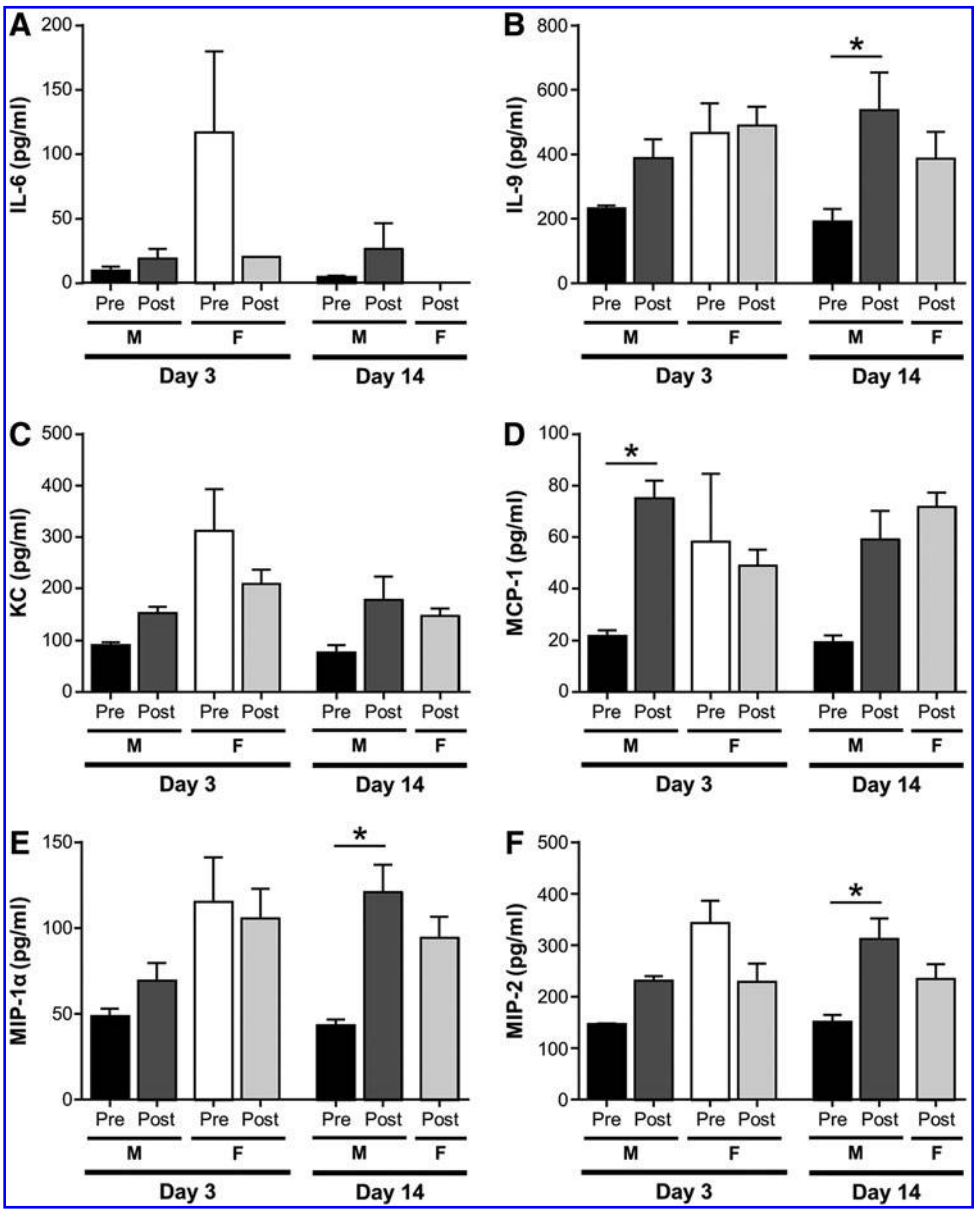

Figure 3. Interleukin-6 (IL-6), IL-9, KC, MCP-1, MIP-1α, and MIP-2 cytokine levels in DKO mice injected with AAV8.TBG.hLDLR. DKO mice were injected i.v. with 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR. Cytokine levels were measured by Luminex in serum samples by the Human Immunology Core at the University of Pennsylvania. Serum from prior to vector injection (pre) and at the time of necropsy on either day 3 or day 14 (post) from both male and female mice were compared for levels of cytokines (**A**) IL-6, (**B**) IL-9, (**C**) KC, (**D**) MCP-1, (**E**) MIP-1α, and (**F**) MIP-2. M, male; F, female. Values expressed as mean $\pm$ SEM (*$p<0.05$; $n=4$/sex/group).

from female mice necropsied on day 14 post vector administration, as samples were not available for testing.

High baseline values were seen for female mice necropsied on day 3 post vector administration (Fig. 3). There were no significant differences in pre- and post-vector administration levels of IL-6 and KC. A significant difference in pre- and post-vector administration levels of MCP-1 was seen for male mice necropsied on day 3 ($p<0.05$). In addition, there was a significant difference in pre- and post-vector administration levels of IL-9, MIP-1α, and MIP-2 in male mice necropsied on day 14 ($p<0.05$). Notably, IL-6 did not significantly increase following vector administration in any of the groups.

NAbs to the AAV8 capsid and T-cell responses to the AAV8 capsid and h*LDLR* transgene

NAbs to the AAV8 capsid were examined for serum samples from day 28 post vector administration taken from mice injected with 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR that were scheduled to be necropsied on day 90. All animals developed a NAb response, with titers that ranged from 1:640 to 1:5,120.

Spleens were collected and splenocytes isolated for IFN-γ ELISPOT analysis from animals that received 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR and were necropsied on day 14 post vector administration. T-cell responses were detected to the AAV8 capsid and h*LDLR* transgene in both male and female DKO mice (Supplementary Table S4). Four female and three male mice were positive for responses to the AAV8 capsid against pool C of the AAV8 capsid library. A low-level T-cell response to the h*LDLR* transgene was detected in one female and one male mouse. This response was only slightly higher than background. Therefore, i.v. administration of 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR induced a low-level T-cell response to the AAV8 capsid and a very low-level T-cell response to the h*LDLR* transgene.

Histopathology

At the time of necropsy, tissues were harvested from all animals for full histopathology. There were no gross pathology findings that were considered to be related to the vectors. In order to facilitate the number of tissues requiring analysis by the pathologist, sections were reviewed in successive rounds, as described below. Initially, all tissues from animals administered with the high dose (6.0×10^{13} GC/kg) of the human and mouse LDLR vectors were reviewed in round 1. In addition, sections from all tissues from mice administered with the vehicle control were reviewed for comparison. Following round 1, four target tissues were identified for round 2 analysis: the liver, lung, brain, and testes. These target tissues were identified due to positive or negative differences between mice administered with the vectors or vehicle control. Round 2 of the analysis was expanded to include sections from the target tissues (the liver, lung, brain, and testes) from mice administered with 7.5×10^{12} GC/kg of the mLDLR vector. The analysis was further expanded in round 3 to evaluate sections from the liver, lung, and testes from mice administered with 7.5×10^{11} GC/kg of the mLDLR vector. The brain was excluded from the round 3 analysis due to a lack of findings in round 2.

This study was performed in DKO mice that develop severe hypercholesterolemia and extensive atherosclerosis to simulate the metabolic and clinical aspects of HoFH in humans. While frank hepatosteatosis is not expected in these animals on a chow diet, background pathology above that observed for C57BL/6 mice was expected due to very high and persistent elevations in serum cholesterol. Therefore, it is not surprising that there were several histopathologic findings in mice injected with the vehicle control, which were subsequently considered to be background findings.

The most important and consistent histopathologic findings following the administration of vectors occurred in the vector target organ, the liver. The incidence and severity of four liver parameters (bile duct hyperplasia, sinusoidal cell hyperplasia, centrilobular hypertrophy, and mononuclear cell infiltration) are summarized in Table 2, which includes all findings for mice injected with vehicle control and indicates the background levels of histopathologic findings for male and female DKO mice. All findings for mice injected with the vehicle control were either minimal or mild, with no moderate or severe findings. It should be noted that 87% of all findings in the control groups were minimal. Only findings in the vector-administered groups that were above the incidence or severity of those seen in the vehicle control–administered groups are presented in Table 2. Due to high background findings in mice administered with the vehicle control, further analysis was focused on any relative findings in mice administered with vectors that were mild or above (mild findings denoted as ** in Table 2). In other words, findings described as minimal were dismissed as being irrelevant, unless associated with a clear dose–response effect or correlated with other findings of liver toxicity.

Mild bile duct hyperplasia and sinusoidal hyperplasia were present above background in a few female mice administered high doses of mLDLR and hLDLR vectors. Findings of centrilobular hypertrophy were present only in male mice administered low and medium doses of the mLDLR vector, but were not seen following administration of high-dose mLDLR or high-dose hLDLR vectors. The finding most relevant to T-cell-mediated immunity, mononuclear cell infiltration, was clearly worst in long-term vehicle control male and female mice and essentially non-existent in any of the vector-administered groups. The decrease in inflammation as a result of vector treatment suggests that there is disease-associated development of mild hepatosteatosis that is prevented by gene therapy, even at low doses. While not summarized in Table 2, there was an increase in the incidence of minimal necrosis of liver in mice injected with 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR on day 180 compared with mice injected with vehicle control.

Analysis of other tissues (including the target tissues of the brain and testes) revealed minimal pathology, most of which was not considered to be related to the vector, because it was mild and also observed in vehicle control–administered animals. Notably, mononuclear cell infiltrates and alveolar histocytosis in some lung tissues in mice injected with vehicle control were minimal to mild and

Table 2. *Summary of liver histopathological findings*

	Bile duct hyperplasia		*Sinusoidal cell hyperplasia*		*Centrilobular hypertrophy*		*Mononuclear cell infiltration*	
	Male	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
PBS	1/7, D3** 1/7, D14* 1/7, D14** 1/7, D90* 1/7, D180*	4/7, D3* 1/7, D14** 1/7, D90* 1/7, D180*	1/7, D90*	5/7, D3* 5/7, D14* 1/7, D90* 3/7, D180*	1/7, D3* 1/7, D3* 2/7, D90** 2/7, D180*	—	3/7, D90* 4/7, D180* 1/7, D180**	5/7, D3* 2/7, D14* 5/7, D90* 5/7, D180* 2/7, D180**
7.5×10^{11} GC/kg mLDLR	—	—	—	—	3/7, D3* 3/7, D14*	—	—	—
7.5×10^{12} GC/kg mLDLR	—	4/7, D14* 3/7, D90* 3/7, D180*	2/7, D14* 2/7, D180*	7/7, D90* 5/7, D180*	3/7, D3* 2/7, D3** 3/7, D14* 1/7, D14** 5/7, D90* 1/7, D90** 5/7, D180*	—	2/7, D14*	—
6.0×10^{13} GC/kg mLDLR	—	2/7, D14* 2/7, D14** 1/5, D180* 1/5, D180**	2/7, D14* 2/7, D180*	6/7, D90* 3/5, D180* 1/5, D180**	4/7, D3*	—	3/7, D3* 6/7, D14* 6/7, D90*	6/7, D14*
6.0×10^{13} GC/kg hLDLR	—	3/7, D90* 3/5, D180*	3/6, D90*	6/7, D90* 1/7, D90** 2/5, D180* 2/5, D180**	—	—	5/7, D14* 1/7, D14** 5/7, D90*	7/7, D14*

At necropsy, tissues were harvested from all animals for full histopathology. The incidence and time points at which findings were identified are listed. All findings are presented for mice injected with PBS, indicating the background levels of histopathological findings for male and female DKO mice. Only findings in the vector-administered groups that were above those seen in the vehicle control–administered groups are presented.

The severity of the pathology is indicated as follows: *mild; **moderate.

D, day.

slighter higher in intensity in some animals that received the vectors, although these were not dose-dependent. A few animals in the 180-day low-dose group were noted to have pulmonary lymphoma upon initial review. However, following peer review, these findings were determined to be non-malignant inflammation, predominantly lymphohistocytic with fewer plasma cells and neutrophils. Therefore, it is not thought that these findings were therapy related.

h*LDLR* mRNA expression

RNA was extracted from liver samples taken at the time of necropsy from mice injected with 6.0×10^{13} GC/kg (day 180) of AAV8.TBG.hLDLR and analyzed for h*LDLR* transcripts by TaqMan qPCR. h*LDLR* transcript levels were compared to RNA levels in liver samples from a previous study where DKO mice were administered with 7.5×10^{12} GC/kg of the same vector lot and mice were necropsied on day 90 post vector administration.[23] RNA levels were only determined for two male and two female mice necropsied on day 90 post vector administration, and for three male and five female mice necropsied on day 180. There was no significant difference in vector-derived RNA ($p>0.05$) in mouse liver harvested on day 90 or day 180 post vector administration, although the dose was eightfold higher for the day 180 time point (Table 3). This suggests some diminution in transgene expression over the three-month interval. Furthermore, there was no significant difference in the expression of h*LDLR* in male versus female mice on day 180 post vector administration. The expression of h*LDLR* mRNA is consistent with the stable reduction in serum cholesterol seen throughout the duration of the study, and supports the claim that there is no difference in transduction and/or transgene expression between males and females.

DISCUSSION

A basic tenet of gene therapy development is to determine the safety and efficacy of a proposed product in non-clinical models before testing in humans. This is challenging with complex biological products, such as gene therapy vectors. For genetic diseases, the most available species for assessing pharmacology are mice that have been genetically engineered to simulate the pathology of the corresponding human condition. In the case of the present product, a mouse model was used that had been engineered to eliminate the expression of

Table 3. *hLDLR RNA expression in DKO mouse liver*

Dose (GC/kg)	*Necropsy time point*	*ID (sex)*	*hLDLR message/μg RNA*	*Average vector GC/μg DNA*
7.5×10^{12}	Day 90	716 (M)	3.43×10^{6}	8.98×10^{5}
		759 (M)	3.48×10^{6}	
		777 (F)	1.18×10^{6}	
		778 (F)	1.78×10^{6}	
6.0×10^{13}	Day 180	790 (F)	3.33×10^{6}	9.96×10^{5}
		717 (M)	1.85×10^{6}	
		720 (M)	5.60×10^{6}	
		788 (F)	4.57×10^{6}	
		787 (F)	1.84×10^{6}	
		731 (M)	5.31×10^{6}	
		782 (F)	1.79×10^{6}	
		781 (F)	9.24×10^{5}	

Mice administered with 7.5×10^{12} GC/kg and 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR were necropsied on day 90 and day 180 post vector administration, respectively. Liver samples from mice administered with 7.5×10^{12} GC/kg of AAV8.TBG.hLDLR were from a previous study with the same vector lot.[23] Following tissue harvest at necropsy, RNA was extracted from liver, and mRNA levels for the h*LDLR* transgene were determined.

endogenous LDLR and Apobec1 (called DKO) in order to develop severe hypercholesterolemia and atherosclerosis on a normal chow diet.[15] Assessment of toxicity is complicated because its etiology can be complex—ranging from host-adaptive immune responses to the vector or transgene product to untoward consequences of ectopic or unregulated expression of the transgene product. In some cases, the underlying pathology of the disease can influence these mechanisms of toxicity and diminish the relevance of wild-type animals. This was a concern for HoFH, where the associated dyslipidemia could lead to inflammation of the liver, which could exacerbate vector-associated liver damage.

This study conducted an evaluation of both pharmacology and toxicity in the context of one integrated experiment in the best available mouse model of the disease. In fact, this general approach is supported by the Food and Drug Administration, as articulated in the recent Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products, where it is stated that "preclinical studies in disease/injury models are encouraged to better define the risk–benefit ratio associated with investigational CGT (cellular and gene therapy) products."[24]

One challenge in the design of this study was concern regarding the use of a vector expressing human LDLR in a mouse. It has been previously shown that binding of human LDLR to mouse-derived ApoB100 (*i.e.*, the key ligand in LDL for the LDLR) was diminished, resulting in decreased *in vivo* activity in mouse models over what would be present in humans.[16] Therefore, evaluating human LDLR activity in a mouse would underestimate the true activity of the vector in humans. To address this issue, two versions of the AAV8 vector were produced: one expressing human LDLR to assess toxicity in the DKO mouse, and one expressing murine LDLR to address pharmacology and toxicity.

Male and female DKO mice 6–22 weeks of age were administered i.v. with one of three doses—7.5×10^{11} GC/kg (low), 7.5×10^{12} GC/kg (medium), or 6.0×10^{13} GC/kg (high)—of AAV8.TBG.mLDLR or 6.0×10^{13} GC/kg of AAV8.TBG.hLDLR. The doses were chosen to reflect the span of the proposed dosing regimen of the clinical trial. An additional cohort of animals received PBS as a vehicle control. Animals were necropsied on day 3, day 14, day 90, or day 180 post vector administration with tissues harvested for comprehensive histopathology examination. Additionally, blood was collected for a comprehensive clinical chemistry panel.

The study was remarkable for little toxicity seen in any of the groups, except for mild and reversible findings in liver pathology associated with the highest dose of the mouse LDLR vector. Of particular note, no significant elevations in important pro-inflammatory cytokines were seen (*e.g.*, IL-6). It was concluded that there were no dose-limiting toxicities, meaning that the maximally tolerated dose is higher than the highest dose that was tested (6.0×10^{13} GC/kg), and that the no adverse effect level was greater than or equal to the middle dose of 7.5×10^{12} GC/kg. This correlated well with the toxicology study conducted in wild-type and $LDLR^{+/-}$ rhesus macaques, which determined the maximally tolerated dose to be $>1.25\times10^{13}$ GC/kg and the no adverse effect dose to be $<1.25\times10^{13}$ GC/kg.[25] A stable reduction of cholesterol that should be clinically meaningful was achieved with the lowest dose of the mLDLR vector that was tested, which means the MED is $\leq7.5\times10^{11}$ GC/kg.

One of the challenges in using genetically engineered strains of mice for assessing safety is disease-associated pathology, such as the steatohepatitis that was seen in older DKO mice, which may confound the interpretation of vector-associated toxicity. Despite this challenge, it was thought prudent to evaluate

safety in the context of steatohepatitis because it may actually influence the severity of vector-induced hepatotoxicity. There was also a concern that constitutive overexpression of LDLR in a hypercholesterolemic animal may lead to acute or chronic lipid overload and exacerbate steatohepatitis. In fact, it was found that vector treatment actually diminished the development of the liver inflammation found in control animals at longer time points. This was presumed to be the result of vector correcting the underlying metabolic drive of the inflammation, which was a massive accumulation of systemic LDL.

ACKNOWLEDGMENTS

We would like to thank Suryanarayan Somanathan (University of Pennsylvania, Gene Therapy Program), Alexandra L. Hanlon (University of Pennsylvania, School of Nursing), and Angela K. Brice (University of Pennsylvania, ULAR) for invaluable contributions, as well as the Human Immunology Core (University of Pennsylvania, Philadelphia, PA). We thank Jennifer Stewart, PhD for editorial assistance with this manuscript. This work was funded by the National Institutes of Health: National Heart, Lung, and Blood Institute (P01 HL059407-17).

AUTHOR DISCLOSURE

J.M.W. is an advisor to REGENXBIO, Dimension Therapeutics, and Solid Gene Therapy, and is a founder of, holds equity in, and has a sponsored research agreement with REGENXBIO and Dimension Therapeutics. In addition, he is a consultant to several biopharmaceutical companies and is an inventor on patents licensed to various biopharmaceutical companies. No competing financial interests exist for the remaining authors.

REFERENCES

1. Gao G, Alvira MR, Somanathan S, et al. Adeno-associated viruses undergo substantial evolution in primates during natural infections. Proc Natl Acad Sci U S A 2003;100:6081–6086.
2. Gao G, Vandenberghe LH, Wilson JM. New recombinant serotypes of AAV vectors. Curr Gene Ther 2005;5:285–297.
3. Wang L, Calcedo R, Wang H, et al. The pleiotropic effects of natural AAV infections on liver-directed gene transfer in macaques. Mol Ther 2010;18:126–134.
4. Wang L, Wang H, Bell P, et al. Systematic evaluation of AAV vectors for liver directed gene transfer in murine models. Mol Ther 2010;18:118–125.
5. Nathwani AC, Reiss UM, Tuddenham EG, et al. Long-term safety and efficacy of Factor IX gene therapy in hemophilia B. New Engl J Med 2014;371:1994–2004.
6. Nathwani AC, Tuddenham EG, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med 2011; 365:2357–2365.
7. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34–47.
8. Goldstein JL, Brown JC. Familial hypercholesterolemia, lipoprotein and lipid metabolism disorders. In: Scriver CR, Beaudet AL, Sly WS, et al., eds. Metabolic Basis of Inherited Disease. Volume II. New York: McGraw-Hill, 1989:1215–1250.
9. Starzl TE, Putnam CW, Chase HP, et al. Portacaval shunt in hyperlipoproteinaemia. Lancet 1973;2:940–944.
10. Starzl TE, Chase HP, Ahrens EH Jr, et al. Portacaval shunt in patients with familial hypercholesterolemia. Ann Surg 1983;198:273–283.
11. Sanan DA, Newland DL, Tao R, et al. Low density lipoprotein receptor-negative mice expressing human apolipoprotein B-100 develop complex atherosclerotic lesions on a chow diet: no accentuation by apolipoprotein(a). Proc Natl Acad Sci U S A 1998;95:4544–4549.
12. Daugherty A. Mouse models of atherosclerosis. Am J Med Sci 2002;323:3–10.
13. Rader DJ, FitzGerald GA. State of the art: atherosclerosis in a limited edition. Nat Med 1998;4:899–900.
14. Rohlmann A, Gotthardt M, Hammer RE, et al. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. J Clin Invest 1998;101:689–695.
15. Powell-Braxton L, Veniant M, Latvala RD, et al. A mouse model of human familial hypercholesterolemia: markedly elevated low density lipoprotein cholesterol levels and severe atherosclerosis on a low-fat chow diet. Nat Med 1998;4:934–938.
16. Kassim SH, Li H, Bell P, et al. Adeno-associated virus serotype 8 gene therapy leads to significant lowering of plasma cholesterol levels in humanized mouse models of homozygous and heterozygous familial hypercholesterolemia. Hum Gene Ther 2013;24:19–26.
17. Kassim SH, Li H, Vandenberghe LH, et al. Gene therapy in a humanized mouse model of familial hypercholesterolemia leads to marked regression of atherosclerosis. PLoS One 2010;5:e13424.
18. Lock M, Alvira M, Vandenberghe LH, et al. Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale. Hum Gene Ther 2010;21:1259–1271.
19. Calcedo R, Vandenberghe LH, Gao G, et al. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J Infect Dis 2009; 199:381–390.
20. Greig JA, Peng H, Ohlstein J, et al. Intramuscular injection of AAV8 in mice and macaques is associated with substantial hepatic targeting and transgene expression. PLoS One 2014;9:e112268.
21. Bell P, Moscioni AD, McCarter RJ, et al. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. Mol Ther 2006;14:34–44.
22. Calcedo R, Vandenberghe LH, Roy S, et al. Host immune responses to chronic adenovirus infections in human and nonhuman primates. J Virol 2009;83:2623–2631.
23. Chen S-J, Sanmiguel J, Lock M et al. Biodistribution of AAV8 vectors expressing human low-density lipoprotein receptor in a mouse model of homozygous familial hypercholesterolemia. Hum Gene Ther Clin Dev 2013;24:154–160.
24. Food and Drug Administration. Guidance for Industry: Preclinical assessment of investigational cellular and gene therapy products November 2013; Availabvle at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.
25. Greig JA, Limberis MP, Bell P, et al. Non-Clinical Study Examining AAV8.TBG.hLDLR Vector-Associated Toxicity in Chow-Fed Wild Type and LDLR+/- Rhesus Macaques. Hum Gene Ther Clin Dev 2017;28:10.1089/humc.2017.014

Received for publication January 21, 2016;
accepted after revision February 28, 2017.

Published online: February 28, 2016.

This article has been cited by:

1. Greig Jenny A. , 1, † Limberis Maria P. , 2, † Bell Peter , 1 Chen Shu-Jen , 1 Calcedo Roberto , 1 Rader Daniel J. , 3, 4 and Wilson James M. 1, * 1Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. 2Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. 3Division of Translational Medicine and Human Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. 4Department of Genetics, University of Pennsylvania, Philadelphia, Pennsylvania. . 2017. Non-Clinical Study Examining AAV8.TBG.hLDLR Vector-Associated Toxicity in Chow-Fed Wild-Type and LDLR+/– Rhesus Macaques. *Human Gene Therapy Clinical Development* **28**:1, 39-50. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental Material]