Immune Responses to Intramuscular Administration of Alipogene Tiparvovec (AAV1-LPL^{S447X}) in a Phase II Clinical Trial of Lipoprotein Lipase Deficiency Gene Therapy

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Abstract

Cellular immune responses to adeno-associated viral (AAV) vectors used for gene therapy have been linked to attenuated transgene expression and loss of efficacy. The impact of such cellular immune responses on the clinical efficacy of alipogene tiparvovec (Glybera; AAV1-LPL^{S447X}; uniQure), a gene therapy consisting of intramuscular administration of a recombinant AAV1 mediating muscle-directed expression of lipoprotein lipase (LPL), was investigated. Five subjects with LPL deficiency (LPLD) were administered intramuscularly with a dose of 1×10^{12} gc/kg alipogene tiparvovec. All subjects were treated with immune suppression starting shortly before administration of alipogene tiparvovec and maintained until 12 weeks after administration. Systemic antibody and T cell responses against AAV1 and LPL^{S447X}, as well as local cellular immune responses in the injected muscle, were investigated in five LPLD subjects. Long-term transgene expression was demonstrated despite a transient systemic cellular response and a stable humoral immune response against the AAV1 capsid protein. Cellular infiltrates were found in four of the five subjects but were not associated with adverse clinical events or elevation of inflammation markers. Consistent herewith, CD8⁺ T cells in the infiltrates lacked cytotoxic potential. Furthermore, FoxP3⁺/CD4⁺ T cells were found in the infiltrates, suggesting that multiple mechanisms contribute to local tolerance. Systemic and local immune responses induced by intramuscular injection of alipogene tiparvovec did not appear to have an impact on safety and did not prevent LPL transgene expression. These findings support the use of alipogene tiparvovec in individuals with LPLD and indicate that muscle-directed AAV-based gene therapy remains a promising approach for the treatment of human diseases.

Introduction

FOR ALMOST TWO DECADES, gene therapy has been recognized as a promising approach but has not been able to be translated into the clinic. On the basis of the recent approval of alipogene tiparvovec (Glybera; AAV1-LPL^{S447X}; uniQure) for the treatment of lipoprotein lipase deficiency (LPLD) in the European Union in October 2012, this picture has started to shift.

Among the different vector systems that are used for gene delivery, recombinant vectors based on adeno-associated virus (rAAV) have been proven as one of the most successful (Kaplitt *et al.*, 2007; Raj *et al.*, 2011). A main concern in using rAAV in the clinic is the impact of pre-

existing and induced immune responses against AAV. Such immune responses are recognized as critical for the safety and efficacy of rAAV; the nature of such immune responses appears related to the target tissue involved. Humoral immune responses to AAV capsid proteins are reported in all clinical studies in which AAV vectors were used to target muscle or liver. However, such antibody responses did not limit efficacy (Brantly *et al.*, 2006, 2009; Rodino-Klapac *et al.*, 2008; Mendell *et al.*, 2009; Gaudet *et al.*, 2013). In some of these studies, cellular immune responses against AAV capsid proteins have also been noted. These responses have been extensively discussed in relation to a study in hemophilia B patients after systemic injection of rAAV that carried an expression cassette for coagulation factor IX

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(FIX). An expansion of CD8+ T cells was observed in parallel with an increase in circulating levels of liver transaminases (Manno *et al.*, 2006; Mingozzi *et al.*, 2007). This T cell response was accompanied by a loss of FIX expression (Manno *et al.*, 2006; Mingozzi *et al.*, 2007), suggesting elimination of the transduced hepatocytes by AAV capsid-directed T cells. In contrast, in a precedent clinical study with alipogene tiparvovec, while intramuscular (IM) administration of alipogene tiparvovec resulted in both humoral responses to AAV capsid in all subjects and moderate and transient cellular responses to AAV capsid in 64% (9 of 14) of subjects, transgene expression was still detectable at 26 weeks, suggesting the persistence of transgene expression despite the immune response (Gaudet *et al.*, 2013). No humoral or cellular immune responses to the LPL transgene were observed (Gaudet *et al.*, 2013). Cellular immune responses were also noted in other clinical studies in which rAAV vectors were administered locally, by IM injection (Brantly *et al.*, 2009; Flotte *et al.*, 2011). However, as with alipogene tiparvovec, these studies showed sustained transgene expression (α 1-antitrypsin AAT) for at least 1 year after delivery, suggesting that in this case the cellular immune responses to the AAV capsid had not eliminated transgene expression (Brantly *et al.*, 2009).

In the present study, five LPLD subjects received alipogene tiparvovec by IM injection. Systemic and local immune responses against AAV1, observed after such administration, were analyzed for their impact on safety and the persistence of LPL transgene expression.

Materials and Methods

Investigational drug

Alipogene tiparvovec (AAV1-LPL^{S447X}; uniQure B.V.) is an investigational drug that consists of a recombinant AAV vector of serotype 1 vector carrying the gene coding for a naturally occurring variant of human LPL (LPL^{S447X}) (Rip *et al.*, 2005). Alipogene tiparvovec was originally produced by Amsterdam Molecular Therapeutics (AMT) B.V., The Netherlands (technology adapted from Negrete and Kotin, 2008), in accordance with good manufacturing practice guidelines. For details on the clinical protocol, see Supplementary Data (Supplementary Data are available online at www.liebertpub.com/hum).

Clinical protocol

CT-AMT-011-02 (ClinicalTrials.gov #CT00891306), an open-label, single-dose study evaluating the safety and efficacy of alipogene tiparvovec (AAV1-LPL^{S447X}), was conducted at the ECOGENE-21 Clinical Research Center, Chicoutimi, Quebec, Canada, in accordance with good clinical practice guidelines (CPMP/ICH/135/95) and the Declaration of Helsinki (Carpentier *et al.*, 2012). Study CT-AMT-011-02 was approved by Health Canada and the Ethics Committee of Chicoutimi Hospital. Subjects participating in study CT-AMT-011-02 were recruited from a database of LPLD subjects held at the participating study centers and coordinated through the ECOGENE-21 Clinical Trial Center, Chicoutimi, Quebec, Canada.

Five subjects with LPLD were exposed to a fixed dose of 1×10^{12} gc/kg alipogene tiparvovec administered as a one-

time series of IM injections into the lower extremities. All subjects were treated with immune suppression starting shortly before administration of alipogene tiparvovec, and this was maintained until 12 weeks after administration. The immune suppression regimen consisted of 3 mg/kg/day cyclosporine A and 2 g/day mycophenolate mofetil. A bolus injection of methylprednisolone (1 mg/kg bodyweight) was given 30 min before alipogene tiparvovec administration.

For follow-up, subjects were monitored at predefined intervals for various clinical parameters, including routine hematology, biochemistry, and immune parameters (Supplementary Table S1). No hematology and routine biochemical assessments were planned after week 12, whereas immunological parameters continued to be assessed after 12 weeks (see Table 1). A biopsy of the injected muscle was scheduled between 14 and 52 weeks after vector administration.

All subjects provided written informed consent. Safety data were monitored by the Academic Medical Center, Amsterdam, The Netherlands.

Muscle biopsy

Muscle biopsies were obtained from the injected muscle (vastus lateralis) and noninjected muscle (tibialis anterior). Biopsies were frozen in isopentane cooled with liquid nitrogen or fixed in formalin. Paraffin sections of formalin-fixed material were prepared for general histology. Six-micrometer-thick frozen sections were routinely stained for the presence of CD3+, CD4+, CD8+, CD20+ lymphocytes and CD68+ macrophages (Miller *et al.*, 1990; Troost *et al.*, 1992; Aronica *et al.*, 2005), major histocompatibility complex HLA-DR and HLA-ABC, Granzyme B, Fas ligand, and FoxP3 (T regulatory cells) (all monoclonal antibodies from Dako). In addition, tissue sections were stained for the presence of LPL^{S447X} using antibody 5D2 (a kind gift from Dr. J.D. Brunzell, University of Washington, Seattle, WA) (Peterson *et al.*, 1992). Neutral lipids were monitored by staining of sections using lipid stain Oil-Red-O.

Per sample, two representative sections were stained for CD3, CD4, CD8, CD20, CD68, HLA-ABC, HLA-DR, Granzyme B, Fas ligand, FoxP3, LPL, and Oil-Red-O and assessed by two investigators independently; a consensus score was obtained.

Detection of vector DNA sequence and LPL protein and activity in muscle biopsies

Muscle biopsies were processed and analyzed by quantitative polymerase chain reaction for the presence of alipogene tiparvovec-derived DNA sequence (by Southern Research Institute). Genomic DNA was isolated by the DNeasy Blood and Tissue Extraction Kit (Qiagen). Primers and fluorescent probe specific for the boundary between the LPL^{S447X} sequence and the WPRE element were used to amplify a sequence specific for alipogene tiparvovec. Sample analysis was performed in a Roche LightCycler 2.0 (software version 4.05). The amount of vector DNA was calculated from a standard curve of alipogene tiparvovec, which was processed using a Viral RNA Extraction kit (Qiagen) and covered a range of 40 to 2.89×10^9 gc. Results were reported as gc per μ g of

TABLE 1. ALIPOGENE TIPARVOVEC-DERIVED VECTOR DNA LEVELS AND LIPOPROTEIN LIPASE EXPRESSION IN MUSCLE BIOPSIES FROM LIPOPROTEIN LIPASE-DEFICIENT SUBJECTS AFTER INTRAMUSCULAR ADMINISTRATION OF ALIPOGENE TIPARVOVEC

Subject	Sample	Biopsy time point	AAV1-LPL ^{S447X} DNA (gc/ μ g)	LPL mass (ng/mg)	LPL activity (nmol/mg/min)
01-001	I	Week 18	130	0.0	0.0
	II		13 ^a	0.2	0.0
01-001	I	Week 52	1,100,000	188.3	54.1
	II		0	0.0	0.0
01-002	I	Week 30	7,800	4.5	0.0
	II		13 ^a	0.0	0.0
01-003	I	Week 14	730,000	49.0	13.0
	II		2,400	1.9	0.0
02-001	I	Week 36	2,500,000	76.5	16.4
	II		0	0.5	0.0
02-002	I	Week 14	770	0.5	0.0
	II		290	0.0	0.0

AAV, adeno-associated virus; LPL, lipoprotein lipase.

From left to right the columns depict (1) subject identity; (2) injected (I) or noninjected muscle (II); (3) time point of biopsy after alipogene tiparvec administration (weeks); (4) levels of vector DNA detected by quantitative polymerase chain reaction in genome copies (gc) per μ g of tissue DNA, (5) and (6) LPL protein mass and LPL activity per mg of protein in tissue homogenate.

genomic DNA. The lower limit of quantitation was 40 gc; the limit of detection was 4 gc.

Muscle tissue homogenates were prepared in homogenization buffer (25 mM NH_4Cl , 5 mM EDTA, 0.04% [w/v], SDS, 0.075% [w/v], Triton-X100, 4.75 U/ml sodium heparin) at a ratio of 100 mg tissue/ml buffer. Tissues were homogenized using a FastPrep F120 tissue homogenizer (ThermoSavant), and homogenates were centrifuged at 14,000 rpm (20,817 rcf) for 5 min at 4°C. Aliquots of the supernatant were frozen at -80°C, to be used for both LPL protein mass and LPL activity measurements. Tissue LPL protein mass was determined using an ELISA procedure (LPL EIA; Markit-M LPL kit from DS Pharma Biomedical Co.). Tissue LPL activity was measured in the laboratory of Dr. J.D. Brunzell (University of Washington) using a radio-labeled triolein-based substrate assay also used to measure LPL activity in postheparin plasma.

Immunological assays

Antibody responses against AAV1 capsid proteins were measured in serum samples using an ELISA procedure. Briefly, AAV1 capsid proteins were immobilized on polystyrene ELISA plates and incubated with the serum samples to be tested. Bound antibodies were detected by a subsequent incubation with conjugated antibodies against human immunoglobulins. The ELISA did not discriminate between IgG subclass antibodies. To identify positive samples, a cutoff level was established using serum samples from 30 healthy volunteers. Antibody responses against LPL^{S447X} were assessed using a similar ELISA procedure; recombinant LPL^{S447X} was used to coat the ELISA plates.

In order to monitor the T cell-mediated immune response in subjects, a one-color interferon gamma (IFN- γ) enzyme-linked immunosorbent spot (ELISpot) assay was developed as described previously (Manno *et al.*, 2006; Mingozzi *et al.*, 2007) and validated at SeraCare Lifesciences. The assay was based on the detection and quantification of IFN- γ secreting cells upon stimulation with AAV1 capsid or LPL^{S447X} antigens. Peripheral blood mononuclear cells (PBMCs) were obtained from the subjects and incubated

with AAV1 (whole virus, 7.5 μ g/ml), the LPL^{S447X} protein (1.7 μ g/ml final concentration), or medium (negative control). Each antigen and control was tested in triplicate. A mix of phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich) served as positive controls. In an IFN- γ ELISpot assay, PMA/ionomycin control usually gives more than 1,000 spot-forming units (SFU)/million cells, unless cell viability is below acceptable levels.

A positive response to an antigen or control was defined by several spots per million PBMCs of at least 50 and at least three times the number of spots measured for the medium-only control.

Results

Monitoring of inflammation after alipogene tiparvec injection

After alipogene tiparvec administration in the presence of immune suppressants (see Materials and Methods for further details), LPLD subjects were monitored for routine hematology and biochemistry. About 12 weeks after administration, there was no obvious change in laboratory parameters, including creatine phosphokinase (CPK), C-reactive protein (CRP), and neutrophil numbers; pre- and postexposure levels of these parameters were nearly all within the normal range. A per-patient summary of these data is given in Supplementary Fig. S1.

The injections were well tolerated. None of the subjects showed signs of persistent local inflammation at the injection sites, such as redness, swelling, warmth, pain, or dysfunction, that could be related to the administration of alipogene tiparvec. Overall, no change in muscle function was clinically observed.

Evidence of long-term LPL^{S447X} transgene expression in injected muscle

Biopsies from injected and noninjected muscle were taken between weeks 14 and 52 after alipogene tiparvec administration (Table 1) and used to monitor the presence of

alipogene tiparvec-derived vector DNA sequence as well as to monitor muscle tissue LPL protein and LPL activity.

Vector DNA was detected in the injected muscle of all five subjects at levels above the lower limit of quantitation of the assay, and above vector DNA levels detected in the corresponding noninjected muscle samples (Table 1). Vector DNA levels in the injected muscles varied considerably between subjects (from 130 to 2.5×10^6 gc/ μ g DNA). Subject 01-001, from whom two biopsies were obtained, showed low levels of vector DNA at week 18 but abundant levels at week 52. This indicates the difficulty to consistently take a sample close enough to the site of injection and, possibly, indicates a limited spread of AAV1 within muscle after injection.

The presence of the LPL^{S447X} protein was investigated by immunohistochemistry and was observed in the injected muscle of four out of the five subjects, while the corresponding noninjected muscle biopsies were negative (Fig. 1). In subject 01-001, the LPL protein was detected at week 52 but not at week 18 (Table 1), supporting the results from the vector DNA analysis.

In line with the immunohistochemistry results, LPL protein mass and activity was detected in the homogenates generated from the biopsies of the injected muscles in four and three of the five subjects, respectively (Table 1). Neither LPL protein mass nor LPL activity was detected or was very low in the corresponding noninjected muscle samples.

The muscle tissue cross sections were also stained for the accumulation of intracellular neutral lipids, as a local marker for LPL activity (Poirier *et al.*, 2000). Lipid accumulation was observed in biopsies from the same four subjects showing positive staining for LPL^{S447X} (Fig. 1 and Table 1).

Induction of antibodies against AAV1 but not against LPL^{S447X}

Before treatment, three out of the five LPLD subjects showed preexisting antibodies against AAV1. After ad-

ministration of alipogene tiparvec, all subjects showed a treatment-emergent anti-AAV1 antibody response. This response became detectable 1–2 weeks after the administration and remained positive throughout the 52-week observation period (Supplementary Fig. S2A). The three subjects positive for AAV1 total antibodies presented preexisting neutralizing antibodies whose titers correlated with total antibodies titers (data not shown) as previously reported for AAV8 in humans (Hurlbut *et al.*, 2010). In contrast, none of the subjects developed an antibody response against LPL^{S447X} (Supplementary Fig. S2B).

Systemic T cell responses against AAV1-specific antigens but not against LPL^{S447X}

PBMCs isolated before and after alipogene tiparvec administration were screened for CD8+ T cells against AAV1 capsid antigens, using an ELISpot assay (Supplementary Fig. S3 and Table 2). Subjects 01-001, 01-003, and 02-002 showed a positive T cell response to AAV1 capsid at a single time point: at weeks 52, 12, and 14 after alipogene tiparvec administration, respectively. Subject 02-001 showed a positive T cell response twice, at weeks 14 and 39, whereas subject 01-002 tested positive repeatedly at weeks 2, 8, 14, and 39 after administration. Overall, transient cellular immune responses were observed in five out of five subjects and did not have clinical consequences as they were not associated with any clinical signs or symptoms such as persistent elevation of blood levels of CRP, CPK, or other inflammation markers (Supplementary Fig. S1).

ELISpot assays were also used to evaluate T cell responses against LPL^{S447X}; however, such responses were not observed.

Local cellular immune responses in the injected muscle

Variable local infiltrates consisting of immune cells were observed in the injected muscle samples, from four

FIG. 1. LPLS447X expression and lipid accumulation in muscle injected with alipogene tiparvec versus noninjected muscle (subject 01-003). *Upper row:* injected muscle showed positive staining for the LPLS447X protein, whereas noninjected muscle was negative. *Lower row:* injected muscle showed positive staining for intracellular lipid (Oil Red O stain). LPL, lipoprotein lipase.

TABLE 2. SYSTEMIC CELLULAR IMMUNE RESPONSES AFTER ALIPOGENE TIPARVOVEC ADMINISTRATION

AAV1-LPL ^{S447X}												
↓												
Patients	Pre-dosis		Post-dosis								Overall assessment	
	Weeks		Weeks									
	Screening	−2	2	4	6	8	12	14	26	39		52
01-001	−		−	−	−	−	−	−	−	−	+	−
01-002	−		+	+	−	+	−	+	−	+	nd	+
01-003	−		−	−	−	−	+	−	−	−	nd	−
02-001	−		−	−	−	−	−	+	−	+	nd	+
02-002	−		−	−	−	−	−	+	−	−	nd	−

nd, not determined.

We considered that a subject developed a T cell-mediated immune response to AAV1 capsid proteins when at least 2 of the 8–9 sampling time points were measured positive (+) in the enzyme-linked immunosorbent spot assay. When only one sampling time was reported positive (+), the T cell response was considered negative.

out of the five subjects. The infiltrates were characterized from moderate in subjects 01-002 and 01-003 to more pronounced in subjects 01-001 and 02-001. There were no infiltrates in the injected muscle of subject 02-002. However, discussed from the perspective of the time of the biopsy, the biopsy of subject 02-002 was taken 22 weeks after the peak of systemic T cell response reached baseline, compared with a few weeks for the other subjects (Supplementary Fig. S3).

The more pronounced infiltrations were characterized by a perivascular to perimysial and endomysial infiltration by B cells (CD20+), macrophages (CD68+), and T lymphocytes (CD3+) (Table 3 and Fig. 2); the T lymphocytes expressed the T cell differentiation marker CD4 (helper T lymphocytes) or CD8 (cytotoxic T lymphocytes) (Figs. 3 and 4). No correlation was established between the presence of infiltrates and the observance of signs of degeneration and regeneration of individual muscle fibers.

Biopsies from the LPLD subjects were also stained for HLA-ABC (MHC class I antigens) and HLA-DR (MHC class II antigen) as indicators for immunological activation and markers for myositis, muscular dystrophies, and in-

flammatory myopathies (Appleyard *et al.*, 1985; Englund *et al.*, 2001). In biopsies with a more pronounced infiltration, some muscle fibers stained positive for HLA-ABC and these fibers were located close to the infiltrates; no staining for HLA-DR was observed on muscle fibers (Table 3). In biopsies with moderate or no infiltration, neither HLA-ABC nor HLA-DR expression was found on myofibers.

Lack of cytotoxicity markers but presence of regulatory marker in the T cell population of muscle injected with alipogene tiparvec

The observation that transgene expression persisted over time, despite the presence of infiltrating CD8+ T cells, triggered monitoring of the cytotoxic potential of these CD8+ T cells. Biopsies from the subjects with a more pronounced infiltration (01-001 and 02-001) were therefore analyzed for expression of Granzyme B and Fas ligand, as markers of cytotoxic capacity (Miller *et al.*, 1990; Walsh *et al.*, 1994; Hamann *et al.*, 1997). In addition, muscle biopsy samples from four subjects who were diagnosed with polymyositis were used as positive controls. None of the CD8+ T cells

TABLE 3. SUMMARY OF IMMUNOHISTOCHEMICAL STAINING OF CRYOSECTIONS OF INJECTED MUSCLE ISOLATED FROM LIPOPROTEIN LIPASE-DEFICIENT SUBJECTS AFTER INTRAMUSCULAR ADMINISTRATION OF ALIPOGENE TIPARVOVEC

CT-AMT-011-02						
Subject	Biopsy, weeks postinjection	Infiltrating cells present in injected muscle tissue				
		CD3	CD68	CD20	HLA-ABC (on fibers)	HLA-D (on fibers)
01-001	18	1+	1+	—	—	—
	52	3+	3+	2+	2+	—
01-002	30	1+	1+	—	—	—
01-003	14	1+	1+	—	—	—
02-001	36	3+	3+	1+	1+	—
02-002	36	—	—	—	—	—

Scoring reflecting the number of infiltrating cells identified using staining of cross sections with cell-specific markers: —, none; 1+, rare; 2+, moderate; 3+, high number. It should be noted that the scores given are arbitrary, simply providing a semiquantitative or relative means of distinguishing between subjects in terms of amount of inflammatory cells observed. As such, a score of 3+ represents the highest score observed in this study.

FIG. 2. Immunohistochemical staining of cryosections of the injected muscle (subject 02-001, 14 weeks). *Upper panels:* CD3+ lymphocytes found within the injected tissue as a large perivascular infiltrate, and more diffuse perimysial and endomysial infiltration. *Lower panels:* staining for CD68+ macrophages within the same area.

FIG. 3. Cytotoxic T cells in the injected muscle. Left panels show injected muscle (subject 01-001) at 400 $\times$ magnification. Right panels show a muscle biopsy of a patient diagnosed with polymyositis. Upper row of each block shows CD8 staining, middle row Granzyme B (GZB) staining, and lower row Fas ligand (FasL) staining. The GZB- and FasL-positive cells stained brown.

FIG. 4. T regulatory cells' presence in the injected muscle. Left panels show injected muscle (subject 01-001) at 400 $\times$ magnification. Right panels show a muscle biopsy of a patient diagnosed with polymyositis. Upper row of each block shows FoxP3 staining, and lower row shows CD4 staining.

observed in the biopsy samples from LPLD subjects 01-001 and 02-001 expressed Granzyme B or Fas ligand, as shown in Fig. 3 for subject 01-001, indicating that the majority of these T cells lacked cytotoxic properties. In contrast, the CD8 $^{+}$ T cells in the biopsies from the subjects with polymyositis stained positive for Granzyme B as well as for Fas ligand.

CD4 $^{+}$ T cells observed in muscle biopsies from LPLD subjects injected with alipogene tiparvovec were further assessed for the expression of the transcription factor FoxP3, as a marker for regulatory T cells. Biopsies from noninjected muscle and from muscle of subjects with polymyositis were tested as controls. As shown in Fig. 4 and Supplementary Fig. S4 for subject 01-001, FoxP3 $^{+}$ /CD4 $^{+}$ T cells were detected in the cellular infiltrates observed in biopsies from LPLD subjects injected with alipogene tiparvovec, but not in the noninjected muscle of the same subjects (Supplementary Fig. S4). FoxP3 $^{+}$ /CD4 $^{+}$ T cells were absent in the muscle biopsy samples from subjects with polymyositis (Fig. 4).

Discussion

The present study shows successful gene delivery and long-term transgene expression in muscle after a one-time IM administration of alipogene tiparvovec—in the presence of immune suppressants—to LPLD subjects. Vector injections were generally well tolerated, and no clinical symptoms such as swelling, pain, or muscle dysfunction were observed.

The persistence of the transgene and transgene expression observed with alipogene tiparvovec (Gaudet *et al.*, 2013) are in agreement with clinical trial results from others, testing IM administration of AAV and showing transgene expression in human muscle being sustained for several years (Manno *et al.*, 2003; Jiang *et al.*, 2006; Brantly *et al.*, 2009). Furthermore, the presence of muscular LPLA activity was associated with significant improvement in plasma chylomicron clearance, a trend that was also observed 52 weeks after gene transfer (Gaudet *et al.*, 2011; Carpentier *et al.*, 2012).

The immunological data show that the transgene product LPL^{S447X} is not immunogenic in LPLD subjects. Neither antibody responses nor T cell responses against LPL^{S447X} were observed.

In line with published data observed with other AAV vectors (Brantly *et al.*, 2006, 2009; Mingozzi *et al.*, 2007; Mingozzi and High 2013) and despite pharmacological immune suppression, sustained antibody responses and transient T cell responses against AAV1 were observed in the subjects administered alipogene tiparvovec. However, the results obtained indicate that these AAV1-specific immune responses did not affect transgene expression. Sustained transgene expression despite T cell responses directed against the capsid antigen has also been reported in other clinical studies targeting muscle, that is, AAT-deficient subjects receiving IM injection of an AAV1-AAT vector (Rodino-Klapac *et al.*, 2008; Brantly *et al.*, 2009) and a study of AAV1- α -sarcoglycan in limb-girdle muscular dystrophy subjects (Mendell *et al.*, 2009). Interestingly, those clinical trials were performed without immune suppression. We are currently investigating if and how the immune suppression regimen used in our study, in the context of muscle delivery, has affected transgene expression.

The presence of preexisting anti-AAV1 antibodies (in three out of the five LPLD subjects) did not affect transgene expression postadministration.

Positive systemic T cell responses to AAV1 capsid were observed at a single time point for subjects 01-001, 01-003, and 02-002, when subject 02-001 showed a positive T cell response twice and subject 01-002 was tested positive repeatedly after administration. In addition, cellular infiltrates including CD8 $^{+}$ T cells were observed in muscle injected with alipogene tiparvovec, as have been reported in other clinical studies with alipogene tiparvovec (Gaudet *et al.*, 2013). Collectively, these responses did not have clinical consequences since they were not associated with clinical signs or symptoms such as elevation of CPK, neutrophil

count, CRP, or other inflammation markers. Although CD8⁺ T cells were present in the infiltrates observed in the injected muscle of four of the five subjects, none were positive for Granzyme B or Fas ligand, which are markers for cytotoxic activity. These data suggest that, unlike T cells present in muscle from subjects with polymyositis, the majority of the T cells observed in muscle injected with alipogene tiparvovec lack cytotoxic properties and therefore likely do not lead to loss of transduced muscle tissue.

Furthermore, in muscle from LPLD subjects injected with alipogene tiparvovec, MHC class I and MHC class II expression on muscle fiber membranes was found to be negligible. This indicates that the muscle fibers were not immunologically active, in contrast to muscle fiber membranes of subjects with polymyositis and muscular dystrophies (Appleyard *et al.*, 1985; Englund *et al.*, 2001). AAV1-transduced myofibers are, therefore, unlikely to have the potential to become CTL targets as they do not have the capability to present sufficient AAV1 capsid peptide on their cell surface. Such a mechanism has been discussed in another AAV1 study targeting the muscle, to explain the lack of CTL response (Brantly *et al.*, 2009).

Regulatory T cells (Tregs) are considered to be key in the counter-regulation of inflammatory reactions. Staining for FoxP3 revealed the presence of FoxP3⁺/CD4⁺ T cells in muscle tissue after injection of alipogene tiparvovec. Previous work involving intravascular delivery of the canine FIX transgene by AAV2/2 in the muscle in a dog model for hemophilia B (Arruda *et al.*, 2005; Haurigot *et al.*, 2010) showed that stable transgene expression was associated with the presence of Tregs. Our findings support the theory proposed by Mays and Wilson (2011) that in case AAV vector administration does not initiate sufficient innate immune activation, a mechanism of passive tolerance may result through ignorance, anergy, or deletion. The data presented in this study support the findings reported by Mueller *et al.* (2013) and suggest that such mechanisms implicating T cell anergy and Tregs may occur after AAV-mediated gene transfer to muscle. Further characterization is required to define how those FoxP3⁺/CD4⁺ T cells are induced upon administration of AAV-based vectors. Yet, their presence, as well as the absence of expression of MHC molecules on the myocytes and the lack of cytotoxic potential of local CD8⁺ T cells, indicates that probably multiple mechanisms contribute to a type of local tolerance toward AAV.

T cell responses are considered a major hurdle in achieving long-term efficacy after AAV-based gene delivery, an assumption mainly based on a study in which AAV2 was used to deliver the gene for FIX to the liver of hemophilia B subjects (Manno *et al.*, 2003; Mingozzi *et al.*, 2007). However, the immunogenicity data found in the clinical studies conducted with AAV-based vectors in human show that immune responses against AAV capsid proteins can vary widely and among others are influenced by the target organ, route of delivery, and dosing schedule. Also, immune modulation was used as part of the Alipogene tiparvovec trial, which could have impacted on the AAV-specific immune responses. Here we report that, in spite of systemic responses against AAV, the local immunological findings from the injected muscle, the lack of clinical side effects, and the course of CPK, CRP, and neutrophil counts upon IM administration of alipogene tiparvovec, all point to

minimal if any immune-mediated damage to the injected muscle. Indeed, we observed sustained transgene expression up to 52 weeks after injection of alipogene tiparvovec in the muscle in spite of systemic and local immune responses. Regarding the potential of AAV1-based gene therapy, we demonstrate safety and efficacy for clinical application targeting the muscle.

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Author Disclosure Statement

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