Human Treg responses allow sustained recombinant adeno-associated virus–mediated transgene expression

Christian Mueller,[1] Jeffrey D. Chulay,[2] Bruce C. Trapnell,[3] Margaret Humphries,[1] Brenna Carey,[3] Robert A. Sandhaus,[4] Noel G. McElvaney,[5] Louis Messina,[1] Qiushi Tang,[1] Farshid N. Rouhani,[6] Martha Campbell-Thompson,[6] Ann Dongtao Fu,[6] Anthony Yachnis,[6] David R. Knop,[2] Guo-jie Ye,[2] Mark Brantly,[6] Roberto Calcedo,[7] Suryanarayan Somanathan,[7] Lee P. Richman,[8] Robert H. Vonderheide,[8] Maigan A. Hulme,[6] Todd M. Brusko,[6] James M. Wilson,[7] and Terence R. Flotte[1,8]

[1]University of Massachusetts Medical School, Worcester, Massachusetts, USA. [2]Applied Genetic Technologies Corp., Alachua, Florida, USA. [3]Cincinnati Children's Hospital, Cincinnati, Ohio, USA. [4]National Jewish Health, Denver, Colorado, USA. [5]Beaumont Hospital, Dublin, Ireland. [6]University of Florida College of Medicine, Gainesville, Florida, USA. [7]Gene Therapy Program and [8]Abramson Family Cancer Research Institute, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA.

Recombinant adeno-associated virus (rAAV) vectors have shown promise for the treatment of several diseases; however, immune-mediated elimination of transduced cells has been suggested to limit and account for a loss of efficacy. To determine whether rAAV vector expression can persist long term, we administered rAAV vectors expressing normal, M-type α-1 antitrypsin (M-AAT) to AAT-deficient subjects at various doses by multiple i.m. injections. M-specific AAT expression was observed in all subjects in a dose-dependent manner and was sustained for more than 1 year in the absence of immune suppression. Muscle biopsies at 1 year had sustained AAT expression and a reduction of inflammatory cells compared with 3 month biopsies. Deep sequencing of the TCR Vβ region from muscle biopsies demonstrated a limited number of T cell clones that emerged at 3 months after vector administration and persisted for 1 year. In situ immunophenotyping revealed a substantial Treg population in muscle biopsy samples containing AAT-expressing myofibers. Approximately 10% of all T cells in muscle were natural Tregs, which were activated in response to AAV capsid. These results suggest that i.m. delivery of rAAV type 1–AAT (rAAV1-AAT) induces a T regulatory response that allows ongoing transgene expression and indicates that immunomodulatory treatments may not be necessary for rAAV-mediated gene therapy.

Introduction

Clinical applications of recombinant adeno-associated virus (rAAV) vectors have shown great promise, including clear signs of clinical efficacy in a number of early-phase clinical trials, including several for Leber congenital amaurosis, Parkinson disease, lipoprotein lipase deficiency, and hemophilia B (1–8). In general, rAAV vectors of various serotypes have been found to be safe and persistent in their effects. However, anti-capsid immune responses have been observed in every trial in which administration was outside of the retina or CNS. These have included the development of neutralizing antibody responses, which may interfere with readministration and the development of effector T cell responses. Since transgene expression in nondividing cells is generally persistent over the long term, readministration may not be a crucial issue if therapeutic levels of protein expression are achieved. However, the significance of anti-capsid effector T cell responses is unclear, and at least some studies have suggested that they target transduced cells and limit the duration of transgene expression (9, 10).

Gene augmentation therapy as a strategy to treat α-1 antitrypsin (AAT) deficiency has been developed over a number of years, beginning with studies of i.m. injection of a rAAV serotype 2–AAT vector (11, 12) and subsequently using a cross-packaged rAAV serotype 1–AAT vector (rAAV1-AAT) in phase I and phase II clinical trials (13, 14) Published results from both of the rAAV1-AAT trials have shown a dose-dependent increase in serum levels of wild-type–specific AAT (M-AAT) levels after i.m. injection, which has persisted in individuals despite the emergence of anti-capsid effector T cells (which have included both $CD4^+$ and $CD8^+$ cells, with $CD8^+$ population cells having markers consistent with cytotoxic T cells) (13, 14). In the most recent report from the phase II trial, persistence of transgene expression was present at 90 days but had declined from an earlier peak value in each subject and was associated with local cellular infiltrates containing both B and T lymphocytes (14). Based on these data, it was not clear whether there would be a continued decline of transgene expression beyond the 90-day time point.

Importantly, longer term follow-up of the same cohorts of subjects for whom the 90-day results were published has shown persistence and an upward trend of M-AAT transgene expression to approximately 3% of the therapeutic target at 12 months after the i.m. administration of the vector. Muscle biopsies showed both persistence of transgene expression and reduced levels of cellular infiltrates. Biopsies were also examined for the presence of cells

Authorship note: Christian Mueller, Jeffrey D. Chulay, James M. Wilson, and Terence R. Flotte contributed equally to this work.

Conflict of interest: Conflict of interest: David R. Knop, Guo-jie Ye, and Jeffrey D. Chulay hold share options in Applied Genetic Technologies Corp. James M. Wilson and Terence R. Flotte are inventors on patents involving AAV that have been licensed to various biopharmaceutical companies. James M. Wilson is a consultant to ReGenX Holdings and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings. In addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.

Citation for this article: *J Clin Invest.* doi:10.1172/JCI70314.

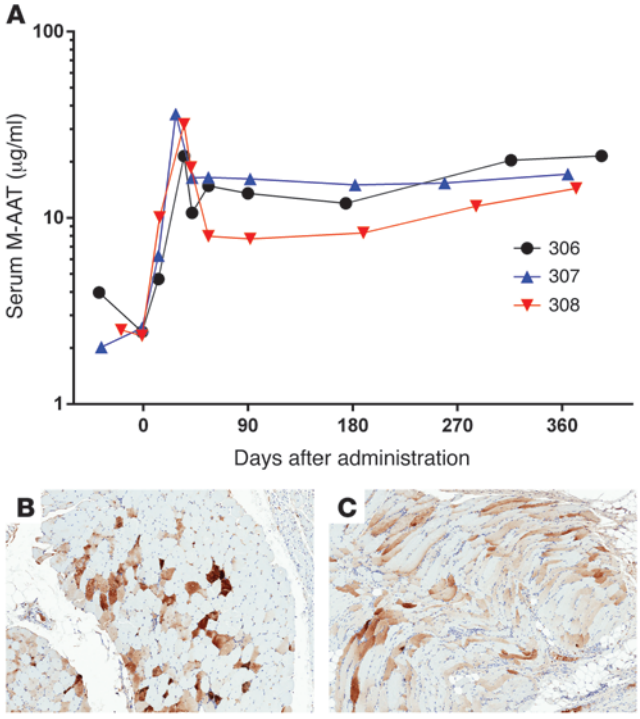

Figure 1
M-AAT expression in skeletal muscle from AAT-deficient human subjects ≥12 months after i.m. injection of rAAV1-hAAT. All subjects in this dose cohort received 6.0×10^{12} vg/kg. The body weight maximum was 90 kg, thus individual doses ranged up to 5.4×10^{14} total vg. The therapeutic target was 572 μg/ml. (**A**) Serum AAT levels detected using a PiM-specific ELISA in subjects 306, 307, and 308. (**B** and **C**) Muscle immunohistochemistry staining for hAAT. Specimens were biopsied from each individual 1 year after rAAV administration and stained for the presence of AAT. Sections show granular reactivity in individual myofibers on cross-section. Immunohistochemistry staining for hAAT in a normal, noninjected muscle is shown in Supplemental Figure 4. Original magnification, ×5.

with T regulatory surface markers ($CD4^+CD25^+FOXP3^+$ colocalization), and many such cells were observed. To determine whether there was a source of antigen for the Tregs, muscles tissue was examined for the presence of adeno-associated virus (AAV) capsid. Confocal analysis with an AAV1 intact capsid-specific antibody revealed the presence of intact capsid at 12 months. These findings, in the absence of any immune suppression, call into question whether anti-capsid T cell responses inhibit the duration of transgene expression after i.m. rAAV vector delivery and suggest that delivery of rAAV to muscle may have clinical utility with modest or no immune suppression. Further studies directly comparing transgene expression levels and duration with or without immune suppression may be informative.

Results

Administration of rAAV1-CB-hAAT by multiple i.m. injections was well tolerated in all subjects. The most frequent adverse events reported in the study were injection site reactions (discomfort, erythema, hemorrhage, or pain) of mild intensity, which occurred in 8 out of 9 subjects. There was one serious adverse event reported. Subject 307, a 51-year-old man with a previous history of emphysema, COPD, and pneumonia with pleural effusion received rAAV1-CB-hAAT (dose 6×10^{12} vector genomes/kg [vg/kg]). At 160 days after injection, he presented to his local hospital with a 3-day history of severe abdominal pain, fever, chills, and diarrhea. An abdominal CT scan showed diverticulitis and probable abscess involving the mid to distal colon. He was treated with metronidazole and levofloxacin for 21 days. At his 9-month and 1-year follow-up visits, he reported that his symptoms were resolved. This adverse event was considered unrelated to study drug administration.

Ongoing transgene expression in patients ≥1 year after i.m. injection of rAAV1-hAAT. The primary end point for biological activity and clinical effectiveness of AAT augmentation therapy in homozygous mutant PI*ZZ patients is an increase in serum M-AAT levels, with the therapeutic target of >11 μM or 572 μg/ml. As demonstrated in Figure 1, M-AAT levels in the high-dose cohort of this study peaked at a mean of 29.8 ± 7.5 μg/ml at 30 days after administration and were sustained at 17.7 ± 3.6 μg/ml (~3% of therapeutic levels) at 12 to 13 months. Lower levels were observed in the lower dose cohorts, as had previously been reported (14), but these were also sustained for 12 to 14 months at levels at or above the levels at day 90 (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70314DS1). Transgene expression was also assessed by immunohistochemical staining of muscle biopsy tissue taken from the injection sites at 1 year after injection. As shown in Figure 1B, transgene expression was present within the injection site at substantial levels as well. Expression did not appear to be influenced by persistence of high levels of anti-AAV–neutralizing antibodies (Supplemental Table 1). No subject developed antibodies to AAT (15) or to the low levels of HSV antigen present in the purified vector (Supplemental Figure 2). Quantitative PCR analysis showed persistent vector DNA in muscle (Supplemental Table 2), rapid disappearance of vector DNA from blood (Supplemental Table 3), and very low and transient levels of vector DNA in semen (Supplemental Table 4).

Cellular infiltrates at the injection site are less prominent but still present at 1 year after injection and predominantly contain macrophages and T cells. Cellular infiltrates consisting primarily of lymphocytes were observed at the injection site at 90 days after administration, as previously reported (14), and were also observed at 1 year after injection in all patients (Figure 2). These infiltrates were decreased compared with observations at the 90-day time point (Supplemental Tables 5 and 6) but were still present in all subjects. IFN-γ ELISPOT responses to AAV capsid library were also decreased as compared with those at the 90-day time point (Figure 2E, Supplemental Figure 3, and Supplemental Table 7). In order to characterize the cellular infiltrates within the injection sites in greater detail, a series of immunophenotyping studies were performed. CD68 staining indicated a consistent presence of macrophages within the infiltrates, which correlated well with the morphology typical of macrophages, as shown by hematoxylin and eosin staining. Despite the waning of IFN-γ response to AAV1 capsid, T lymphocytes within the biopsy samples were identified using CD3 staining, with substantial proportions of $CD4^+$ and $CD8^+$ cells present among the $CD3^+$ population.

Persistence of an oligoclonal population of T cells in the muscle. PBMCs (before and 1 month after injection) and muscle biopsies (3 months and 1 year after injection) from subject 306 were further analyzed for the presence of T cell clones by deep sequencing of the vari-

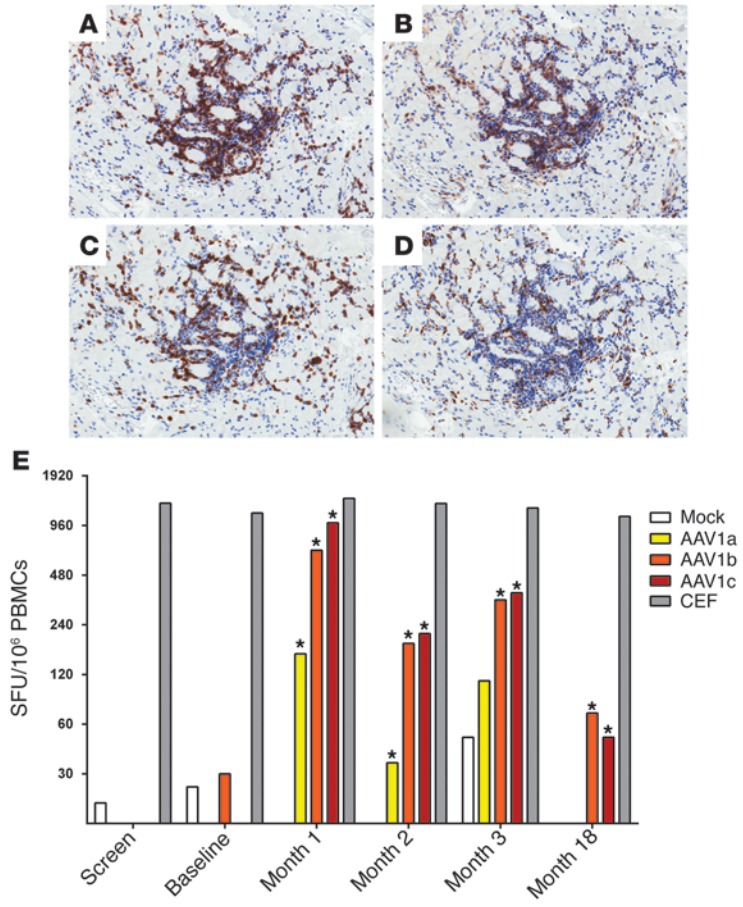

Figure 2

Persistence of lymphocytic infiltrates in muscle more than 1 year after administration (**A**) Immunohistochemistry showing a nidus of infiltration with CD3+ T cells. (**B**) CD4-immunoreactive T cells comprise a substantial subset of the total lymphocytic infiltrate. (**C**) CD8-immunoreactive T cells are also present in the infiltrate. (**D**) CD68+ macrophages are present within and around the lymphocytic infiltrates. Original magnification, ×10. (**E**) Representative time course of IFN-γ ELISPOT (subject 307) responses to pools of AAV1 capsid peptides or controls. PBMCs were obtained at screening, baseline, and 1, 2, 3, and approximately 18 months after vector administration and were stimulated with one of three pools (**A**–**C**) of AAV1 capsid peptides (15-mers overlapped by 10 amino acids) or with a positive control peptide pool (CEF). SFC, spot-forming cells. Positive responses to AAV1 capsid peptides are indicated by *.

able CDR3 region of the TCRβ-chain (TRBV). We compared the repertoire of T cell clones present before and after vector administration to evaluate the emergence of new clones. T cell clones that emerged only after vector administration were designated as infinity clones. Using this analysis, 223 infinity T cell clones were found in blood. Interestingly, 87 out of the 223 infinity T cell clones were also present in muscle 3 months after vector administration, with some clones present at a frequency as high as 6.4% (Figure 3A). Analysis of the TRBV usage in muscle indicated a bias toward clones belonging to the TRBV7-9 family, which also included those T cell infinity clones with the highest frequencies (Figure 3B). The numbers of infinity clones in muscle contracted from a high of 87 at 3 months to 7 at 1 year after vector administration, with frequencies <3%. Interestingly, 1 year after vector administration, only 1 out of the 7 infinity clones belonged to the TRBV7-9 family. Instead, TRBV usage was now dominated by the TRBV18 family (Figure 3C). Our analysis indicates that, after vector administration, an oligoclonal population of T cells emerged in blood that migrated to the site of injection. It should be noted that our analysis of the CDR3 region does not allow classification of the individual clones into T cell subtypes.

Identification of cells with Treg markers at the injection site. The presence of CD8+ T cells at the site of injection at both 3 and 12 months was suggestive of a peripheral cytotoxic T cell (CTL) response to AAV capsid epitopes. However, under these circumstances, the persistence of transgene expression at high levels 1 year after injection was perplexing and more so in the face of decreased IFN-γ responses to AAV1 capsid. In order to determine whether the CD4+ T cell population might be exerting a regulatory effect over the CD8+ T cell population within the muscle, we performed confocal microscopy with immunofluorescent staining for the characteristic CD4+ T regulatory markers, i.e., for colocalization of a CD4 signal with that of CD25 and FOXP3. As shown in Figure 4, A–F, a substantial proportion of cells staining for CD4 were also CD25+ and FOXP3+. Conversely, a substantial proportion of CD25+ and FOXP3+ cells were also represented in the CD4+ population. These results were consistent with an in situ T regulatory response enabling persistence of AAT transgene expression in the face of resident CD8+ T cells. A more quantitative analysis of T cells and Tregs was performed by analyzing the Treg-specific demethylated region (TSDR) within the *FOXP3* gene by qRT-PCR of bisulfite-converted DNA from muscle biopsies. Importantly, this assay can differentiate natural Tregs from other cells that may be transiently expressing FOXP3 by measuring unique epigenetic modifications at the *FOXP3* locus present only in Tregs. Along with cadaveric muscle controls, muscle biopsies from a patient from the middle-dose cohort (subject 304) and one from the high-dose cohort (subject 308) were analyzed for epigenetic TSDR demethylation at 3 and 12 months after vector administration. The overall CD3+ epigenetic analysis confirmed histologic findings that showed a clear influx of T cells that stained for both CD8 and CD4. Moreover, this assay was able to detect a consistent time-dependent decrease in muscle inflammation in both patients, as determined by the decrease in overall number of CD3+ T cells from 3 to 12 months (Figure 4G). More importantly, by quantifying the TSDR, we were able to confirm that the muscle had a substantial proportion of Tregs in situ; in fact, at 3 months close to 5% of all the cells in the muscle were Tregs (Figure 4H). While it was evident that there was a decrease in the T cell infiltration from 3 to 12 months, the proportion of all CD3+ T cells that were Tregs remained close to 10% across time in both patients (Figure 4I).

Tregs are activated by AAV capsid. The presence of Tregs in the muscle that we described above may offer insight into the persistence of AAT transgene expression despite the presence of CD8+ T cells and IFN-γ–positive ELISPOT responses against the AAV capsid. To further determine whether the Treg responses were specific to AAV capsid, we examined cell surface expression of activation markers in Tregs after antigen stimulation. To evaluate this, PBMCs from 8 out of 9 subjects were stimulated with AAV and AAT peptide

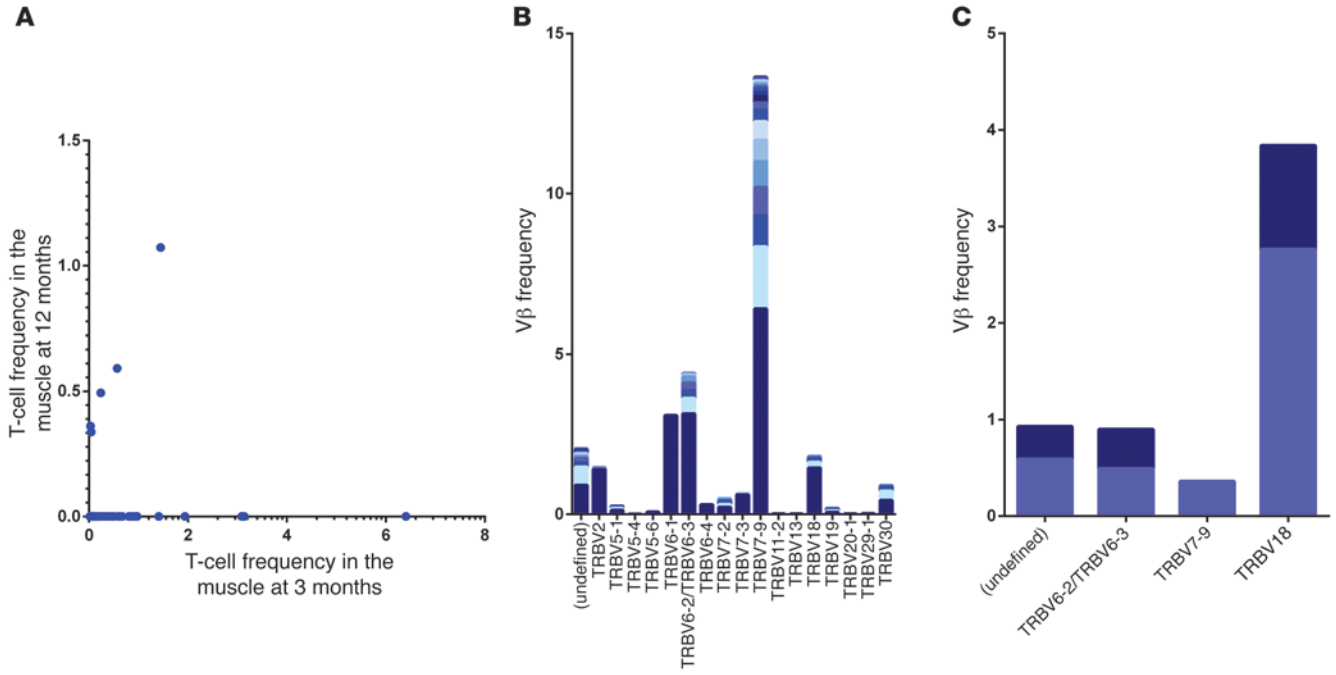

Figure 3

TCR β-chain deep sequencing analysis in subject 306. (**A**) Frequency of T cell clones with a frequency greater than 0.005% that were present in blood only after gene transfer and in injected muscle tissue after 3 months and 1 year of vector administration. (**B**) Frequency of T cell clones arranged by specific Vβ chain in muscle biopsies (**B**) at 3 months and (**C**) after 1 year of vector administration. Colors in each stacked bar indicate unique T cell clones.

libraries in vitro. Two days after stimulation, the cells were analyzed by flow cytometry, Tregs were gated by coexpression of the transcription factors FOXP3 and Helios, and all subsets were then analyzed for expression of CD25 and OX40 as indicators of antigen-specific activation, as previously described (16). The data were then plotted as percentage of cells with $CD25^{+}OX40^{+}$ expression above what was seen in conventional T cells with CEFT stimulation. As shown in Figure 5 and Supplemental Figure 5, Tregs present in the PBMCs were generally activated when stimulated with AAV capsid peptide pools. In contrast, activation of Tregs by AAT peptide pools was seen in only 3 patients (subjects 302, 306, and 307), and, in each case, the frequency of activated Tregs after stimulation by AAT peptides was much lower than that after stimulation by AAV capsid peptides.

Presence of AAV capsid antigen within the injected myofibers. Since the majority of subjects demonstrated peripheral T cell reactivity to epitopes within the AAV1 capsid rather than to AAT, we sought to determine why such T cells would still be residing within the injected muscle at 12 months after injection. The most obvious hypothesis seemed to be that AAV1 capsid protein was persistent within the injected muscle at that time point. In order to test this hypothesis, we performed immunofluorescent staining for intact AAV1 capsids on the muscle biopsies at 12 months after injection. As demonstrated in Figure 6, intact AAV1 capsids were present within myofibers of the muscle, mostly in distinct foci at the perinuclear region.

The long-lasting presence of rAAV1 capsid may in some ways mimic a chronic viral infection, which has been shown to induce Treg responses (17) along with what has been termed "exhausted" T cells. The costimulatory pathways of programmed death-1 (PD-1 also known as CD279) and its ligand PD-L1 (CD274) have been implicated in regulating the balance of T cell activation and tolerance in chronic viral infection and immune-mediated tissue damage by delivery inhibitory signals (18–20). In this way, the PD-1/PD-L1 pathway contributes directly to T cell exhaustion during chronic infections, as it remains expressed at high levels in the setting of persistent antigen (21). In order to determine whether this pathway could be active in the injected muscle, we stained muscle sections for PD-1 and PD-L1. As shown in Figure 7, PD-1 was mostly expressed by cells found in the inflammatory infiltrates of the muscle. PD-L1 was also observed within these foci, but its distribution was more widespread than PD-1. Infiltrates in muscle sections from all patients at both time points were positive for both PD-1 and PD-L1.

Discussion

Although AAT augmentation therapy can achieve effective serum levels of AAT, this therapy is not ideal due to the need for weekly intravenous infusions, high annual costs, and insufficient availability of product to treat all persons currently diagnosed with severe AAT deficiency, resulting in low physician motivation to accurately diagnose the vast majority of patients who remain unrecognized. If treatment with a rAAV vector expressing AAT can achieve similar serum AAT levels, it would provide a more readily available and convenient, potentially 1-time, treatment option.

In the 12-month follow-up period of this phase II gene transfer study for AAT deficiency, we observed persistent gene expression in the absence of immune suppression. The earlier time points within this study seemed to mimic those seen in other trials of both hepatic delivery and muscle delivery of rAAV vectors, with an anti-

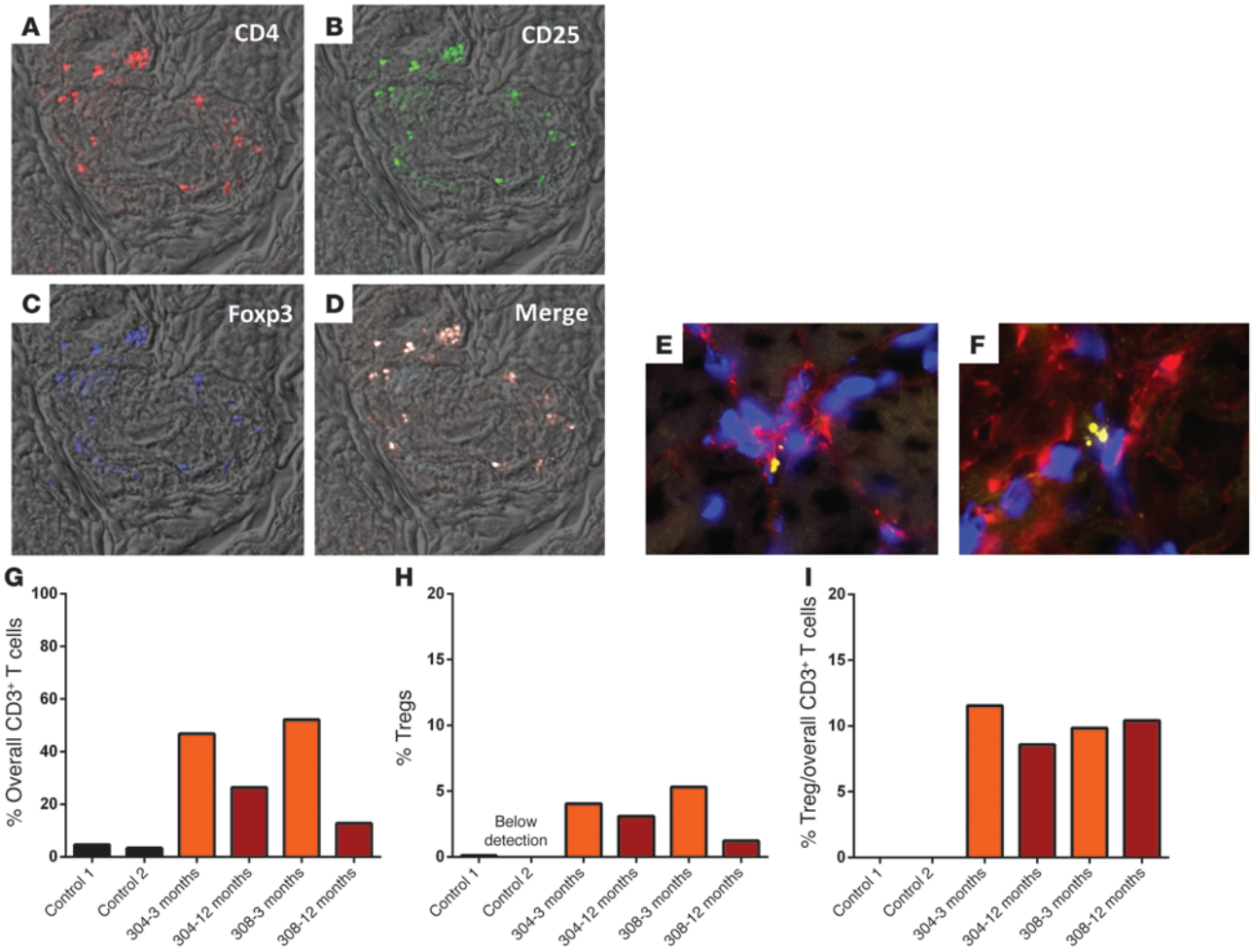

Figure 4

In situ detection of Tregs at 12 months after vector administration. (**A**–**C**) Formalin-fixed, paraffin-embedded muscle biopsies from patients were stained with antibodies specific for the cell surface markers CD4 and CD25 and the transcription factor FOXP3. Original magnification, ×10. (**D**) The merged image shows the presence of Tregs in muscle tissue. Flash frozen muscle sections were also stained with DAPI (blue), CD4 (red), and FOXP3 (yellow) for higher resolution of FOXP3 localization. **E** and **F** show higher-magnification images of $FOXP3^+CD4^+$ cells in the muscle biopsies. Original magnification, ×10 (**A**–**D**); ×40 (**E** and **F**). (**G**–**I**) Analysis of the FOXP3 TSDR. Epigenetic detection was used to quantify total (**G**) $CD3^+$ cells and (**H**) Tregs in the muscle biopsies of patients 304 and 308 and (**I**) the proportion of muscle T cell infiltrates that that were Tregs.

capsid effector T cell response within the first 30 days after administration, resulting in cellular infiltration at the injection sites, a transient rise in creatine kinase, and a partial decline in transgene expression. Remarkably, despite a persistence of peripheral anti-capsid T cells and local cellular infiltrates, transgene expression persisted for more than 1 year after vector administration, with no sign of diminution of expression levels. To the contrary, an upward trend was seen in each of the 3 patients in the cohort receiving the highest dose (those receiving doses of 6.0×10^{12} vg/kg). These findings call into question whether the cellular infiltrates present within the muscle were actually functionally cytotoxic in nature. A possible finding supporting this is the evidence of an expansion of Tregs found in situ. These results generally raise expectations about the long-term utility of muscle-directed rAAV gene therapy. The sustained levels seen here (17.7 ± 3.6 μg/ml) were relatively high in absolute terms for protein expression but were lower than the very high target level needed for correction of AAT deficiency, at 3% of the target of 572 μg/ml.

In comparing this study to other recent studies of rAAV-based gene therapy, it is important to point out that while there are important commonalities in the findings, none of the studies were designed precisely in the same manner as this one. All studies of liver and muscle delivery of rAAV have shown some evidence of anti-capsid T cell responses, often with some indication of cellular toxicity (elevation of transaminases or CK), and most showed at least some diminution of gene expression. In early studies of liver-directed therapy, patients were followed without immune suppression, but more recent studies have included some element of immune suppression, with initiation of such therapy either at the time of vector administration or in response to evidence of potential cytotoxicity manifested by elevation of serum enzymes. Thus, our study provides the unusual perspective of following the course after i.m. delivery without any additional intervention.

Clearly, the current study does not address the question of whether transgene expression might have been significantly higher if immune suppressive or antiinflammatory drugs had been used.

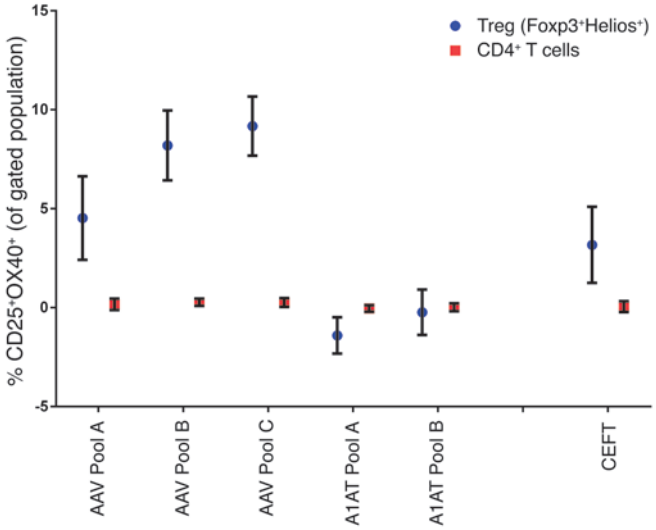

Figure 5
Antigen-specific activation of Tregs. PBMCs were stimulated with AAV, AAT peptide pools, or 1 μg/ml CEFT peptide. Cells were harvested at 48 hours after activation and gated for live $CD4^+$, $FOXP3^+$, and $Helios^+$ cells and then subgated for activation markers $OX40^+$ and $CD25^+$. Lymphocytes were gated on forward and side scatter gates. Live $CD4^+$ T cells were subgated for analysis of specific subsets as follows. Tregs were gated by coexpression of the transcription factors FOXP3 and Helios. Conventional T cells were gated as $CD4^+FOXP3^-Helios^-$. All subsets were then analyzed for expression of CD25 and OX40 as indicators of antigen-specific activation. The data are plotted as activation above $CD4^+$ CEFT stimulation. Data are shown as an average for all 8 samples ± SEM.

In the recent report by Nathwani et al. (6), in a liver-directed hemophilia B trial, a modest immune modulation (60 mg prednisolone daily with tapering and discontinuation over 4 to 7 weeks) was sufficient to enhance gene expression and decrease transaminase elevation. This is very promising, since it could readily be incorporated into an AAT gene augmentation trial without a large increase in the risk to the study volunteers.

The current study's results also do not predict whether or not further increases in the dose would continue to produce similar responses at proportionally higher levels. It was very encouraging that there was a linear dose-response relationship within the dose range used in this trial. However, since the current trial involved 100 individual i.m. injections of 1.35 ml each, further increases in the dose will likely require some form of regional vascular delivery (22, 23). This could potentially alter both the nature of distribution of the vector among the myofibers and the relative exposure of lymphoid tissues to the vector material. How this would affect the results is difficult to predict, although some preclinical data suggest that the amount of transgene expression observed for any given dose of vector could be higher with a regional vascular delivery method (23, 24).

It has been generally accepted that, after cellular uptake, unprocessed rAAV vector capsid would be mostly degraded by the proteasomal machinery; however, in this study, we show the first published evidence of the persistence of intact capsid up to a year after administration in humans. This finding is consistent with an earlier study that documented the detection of rAAV particles up to 6 years after administration in the retina of dogs and nonhuman primates (25) and may explain why Tregs would continue to reside within the injected muscle months after administration. This has important consequences for the design of future clinical trials, since the presence of viral antigens may not be as transient as once believed, thus influencing the timing and length of immunosuppressive strategies. Even more importantly, the persistence of capsid may mimic a chronic viral infection, which ultimately may limit excessive inflammation and allow persistence of transduced cells, as natural Tregs develop to protect against overexuberant immune responses and bystander killing of untransduced cells. It is known that natural Tregs ($CD4^+CD25^+FOXP3^+$) arise in the thymus during development and are thought to possess T cell receptors specific for self antigens (26). However, it has also been well documented that these cells also suppress immune response to infectious agents. The mechanism by which this happens is still unclear, and it is largely unknown whether these natural Tregs require priming to recognize a viral or foreign antigen. Possible mechanisms that have been suggested include

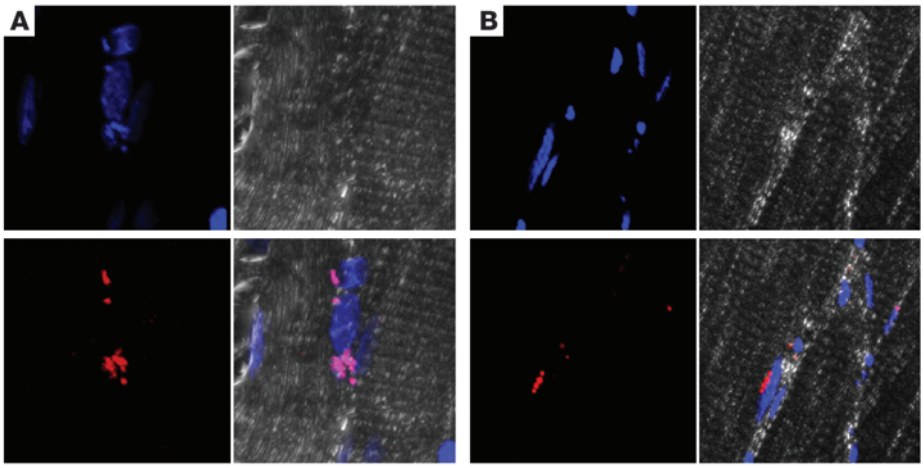

Figure 6
In situ detection of intact AAV1 capsid in the muscle at 3 and 12 months after vector administration. Muscle biopsies at (**A**) 3 months and (**B**) 12 months after administration of the vector were stained with DAPI (blue, top left) and an antibody specific for AAV1 intact capsid (red, bottom left). The top right image of each panel shows a DIC image, and the bottom right image of each panel is a merged image. Original magnification, ×40.

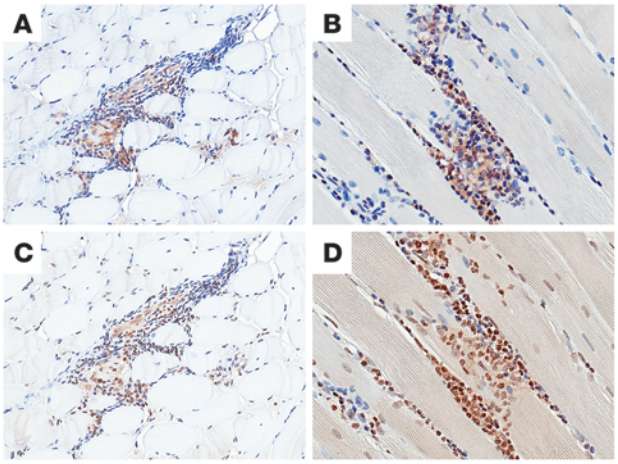

Figure 7
Representative in situ detection of PD-1 and PD-L1 in muscle lymphocytic infiltrates. Muscle biopsies were stained for (**A** and **B**) PD-1 or (**C** and **D**) PD-L1. Sections show positive staining for PD-1 and PD-L1 in lymphocyte infiltrates. Original magnification, ×10 (**A** and **C**); ×20 (**B** and **D**).

the nonspecific activation of Tregs through Toll-like receptor signaling or Treg stimulation by cross-reactive epitopes (27, 28). The data presented here suggest that the Treg response is at least in part responsive to AAV capsid, as was seen by the capsid-induced activation. Taking into consideration that the Tregs detected in muscle had a demethylated TSDR and the capsid activation was observed among Tregs that were Helios$^+$, it is likely that these are natural Tregs. Thus, the capsid-specific activation is not necessary a de novo Treg response but could also be explained by an expansion of a preexisting repertoire. While the mechanism remains elusive, it is clear that virus-specific natural Tregs allowing viral persistence have been observed in humans with chronic hepatitis C virus (29, 30). Additionally, the expansion of Tregs in general has been also associated with the chronicity of HIV and papillomavirus infections (31–33). Thus, in this regard, the persistence of rAAV capsid and its epitopes could be mirroring these chronic viral infections. Interestingly, expression of PD-1 and PD-L1, which is associated with chronic viral infection and exhausted T cell responses (18–20), was also observed in many of the inflammatory foci of the rAAV-injected muscles. Consistent with our findings, not only does the PD-1/PD-L1 pathway limit T cell stimulation, but it also promotes the differentiation and maintenance of FOXP3$^+$ Tregs. Finally, it should also be pointed out that while our data show an expansion of Tregs in the muscle, this is not necessarily the site in which the response arose. The levels of vector genomes in the blood after administration suggest that a substantial amount of vector may have transduced the liver or spleen, and it is possible that one of these sites could be playing a role in the induction of the Treg response.

Taken together, the results presented here are highly encouraging for the future of muscle-directed rAAV gene therapy. Persistent anti-capsid T cell responses were both well tolerated, in terms of clinical safety parameters, and did not inhibit persistent transgene expression at 1 year, and the potential mechanism for this was identified, namely, the presence of a T regulatory response. Thus, the anti-capsid responses might be considered to be a relatively manageable aspect of this therapy, at least within the dosage and time ranges tested. Indeed, some targets for serum replacement of other proteins might well have been reached with the methods used in the current study. AAT deficiency remains a very high target for full replacement, and many questions remain, as higher doses and immune suppression are contemplated for future trials. However, given the scalability of rAAV vector production (34), the availability of clinically tolerable limb infusion methods (22, 23, 35, 36), and the relatively modest levels of immune suppression that may have a salient effect (6), it would seem that trials designed to achieve therapeutic levels of serum AAT should be feasible.

Methods

Vector production and characterization. As described in the 3-month interim report (14), the rAAV1-CB-hAAT vector was identical to that used in a previously published phase I clinical trial (13), except that it was made using a recombinant HSV complementation system (37). It was produced in compliance with current good manufacturing practice at SAFC Pharma and characterized using product-specific assays (37).

Study design and conduct. As indicated in the 3-month interim report, this was a nonrandomized, open-label, multicenter, sequential, 3-arm, phase II clinical trial evaluating the safety and efficacy of administration of rAAV1-CB-hAAT conducted, under an IND with approval of University of Massachusetts Medical School and Cincinnati Children's Hospital institutional review boards and institutional biosafety committees, and in accordance with the tenets of the Declaration of Helsinki. This study is registered at Clinical Trials.gov (NCT01054339). Nine subjects (three per cohort) received i.m. doses of rAAV1-CB-hAAT (6×10^{11}, 1.9×10^{12}, or 6×10^{12} vg/kg body weight). The rest of the study design was reported earlier (14).

Laboratory assessments. Antibody assays, AAT serum levels, ELISPOTs, and other laboratory assessments were done as previously described in the 3-month interim report (14).

Confocal microscopy. Formalin-fixed, paraffin-embedded tissue slides were sectioned and deparaffinized. The membranes were dissolved with permeabilization buffer (Dulbecco's PBS with 0.2% Triton X-100) for 2 hours, followed by 3 washes with PBS-Triton wash buffer (PBS with 0.05% Triton X-100) for 5 minutes each. The slides were then blocked with blocking buffer (10% normal bovine serum in Triton wash buffer) for 2 hours, followed by incubation with an antibody cocktail (mouse anti-human FOXP3 Alexa Fluor 488, BD Pharmingen catalog no. 560047; mouse anti-human CD4 APC, BD Pharmingen catalog no. 555349; and mouse anti-human CD25 PE, BD Pharmingen catalog no. 555432) diluted 1:2,000 in Triton wash buffer with 5% normal bovine serum in the humidity chamber for 2 hours at 37 degrees. After incubation the slides were washed 3 times with PBS-Triton wash buffer, followed by 3-minute PBS washes. The slides were then immediately mounted with Permount (Fisher SP15-500), followed by microscopy.

For frozen muscle, 8-μm sections were stained with Dako Autostainer-Plus. The slides were fixed in cold acetone (–20°C) for 10 minutes and then air dried for 1 hour. Slides were washed twice with TBS-Tween 20, pH 7.6, for 5 minutes, followed by a 1-hour room temperature incubation with anti-human FOXP3 purified rat (clone PCH101, catalog 14-4776-82, eBioscience) at a 1:20 dilution with Antibody Diluent (catalog S0809, Dako) and anti-human CD4 (clone 4B12, catalog M7310, Dako). After primary antibody incubation, slides were washed again with TBS-Tween 20, pH 7.6, followed by a 1-hour incubation at room temperature with a secondary antibody (Alexa Fluor 546 goat anti-rat IgG [H+L], catalog A11081, Life Technology), diluted 1: 200, and Alexa Fluor 568 goat anti-mouse IgM (μ Chain) (catalog A11081, catalog A21043, Life technology) diluted 1: 200. Finally, slides were washed twice as above and mounted with ProLong Gold Antifade Reagent with DAPI (P-36931, Life Technology).

For AAV1 capsid staining, an antibody against AAV1 intact particles, the anti-AAV1 capsid antibody (catalog no. 03-610150, American Research Products Inc.), was diluted 1:20, followed by incubation with a secondary antibody, goat anti-mouse IgG (diluted 1:2,000), labeled with Alexa Fluor 514 (catalog no. A31555, Life Technologies). The slides were washed 3 times with PBS-Triton wash buffer for 5 minutes between applications of the 2 antibodies.

Bisulfite TSDR assay. Epigenetic T cell analysis was performed as previously described (38). Briefly, DNA was extracted from flash-frozen muscle tissue using a DNeasy Blood & Tissue Kit (Qiagen). Bisulfite conversion was performed on 2 μg genomic DNA using the EpiTect Bisulfite Kit (Qiagen). Quantitative PCR was performed using EpiTect-MSP (Qiagen).

TCRβ deep sequencing analysis. Patient frozen muscle biopsies or PBMCs underwent deep sequencing by Adaptive Biotechnologies based on a multiplexed PCR method designed to amplify all possible rearranged genomic TCRβ sequences. TCRVβ usage was also determined for each clone, as previously described (39, 40). Output data were normalized for PCR bias and then analyzed using Clone Tracker software provided by Adaptive Biotechnologies. Unique T cell clones were tracked in frequency over time in both the peripheral blood and muscle tissues.

Muscle biopsies were not collected from subjects in this study prior to vector administration, but T cell clones in PBMCs before and after AAV administration were available for analysis. Therefore, only clones present in blood after vector administration and identified at a threshold frequency of greater than 0.005% were considered. The presence of these clones was evaluated in muscle. Dot plots were prepared by plotting frequency of individual clones at 2 time points. Clones that were not detectable above the 0.005% threshold at blood baseline but were present after treatment were plotted as "infinity clones."

Muscle tissue immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Briefly, 4-μm serial sections were deparaffinized and incubated with 3% H_2O_2/methanol to block endogenous peroxidase activity. Then, sections were treated by different antigen retrieval (Supplemental Table 8). After that, sections were blocked with Sniper (Biocare Medical) to reduce nonspecific background staining. Sections were incubated with primary antibodies (Supplemental Table 8) at room temperature for 1 hour or 4°C overnight individually. Then, sections were incubated with Mach2 rabbit HRP polymer (Biocare Medical) or Mach2 mouse HRP polymer for 30 minutes. Staining was visualized with DAB chromogen (Biocare Medical).

Treg activation flow cytometry assay. PBMCs were isolated by Ficoll density gradient centrifugation from sodium heparinized blood samples upon receipt, frozen in FBS with 10% DMSO, and stored in vapor-phase liquid nitrogen until the time of assay. Frozen PBMCs were thawed rapidly at 37°C, washed, and cultured in complete RPMI 1640 (Cellgro) supplemented with 5 mM HEPES, 2 mM L-glutamine, 50 mg/ml penicillin/streptomycin (Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 5 mM nonessential amino acids, 5 mM sodium pyruvate (Mediatech), and 10% FBS (KSE Scientific) for 48 hours in the presence of indicated AAV, A1AT peptide pools, or 1 μg/ml CEFT peptide pool (JPT Peptides). Cells were harvested at 48 hours after activation, stained with a viability dye (LIVE/DEAD, Invitrogen), and then fixed and permeabilized for further staining of surface and intracellular markers of activation and memory: CD4, CD25, OX40, CD45RO, FOXP3, and Helios (Biolegend). Data were analyzed using Flowjo software (Treestar) and GraphPad Prism. Lymphocytes were gated on forward and side scatter gates. Live $CD4^+$ T cells were subgated for analysis of specific subsets as follows. Tregs were gated by coexpression of the transcription factors FOXP3 and Helios. Conventional T cells were gated as $CD4^+FOXP3^-Helios^-$. All subsets were then analyzed for expression of CD25 and OX40 as indicators of antigen-specific activation (16).

Statistics. All ELISPOT data were compared using an unequal variance 2-tailed Student's *t* test. Differences were considered statistically significant if $P < 0.05$. Statistical evaluation using ANOVA was used to compare the ratio of ELISPOT responses at 3 and 12 months.

Study approval. Ethical permission for these studies was obtained from the Institutional Review Boards of the University of Massachusetts Medical School and Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA. The study was conducted under the US Food and Drug Administration Center for Biologics Evaluation (BB-IND 12728) and the NIH Office of Biotechnology Activities protocol no. 0910-1002. The National Heart, Lung, and Blood Institute (NHLBI) Data and Safety Monitoring Board for Gene and Cell-Based Therapies monitored this study. Written informed consent was obtained from each subject following detailed explanation of the procedures.

Acknowledgments

We would like to acknowledge the NHLBI of the NIH for funding (R01-HL69877 to T.R. Flotte) and for providing toxicology testing services (RSA 1056) through the Gene Therapy Resource Program and the Alpha One Foundation for assistance in the recruitment of study volunteers. This study was also supported in part by the Office of Orphan Products Development, US Food and Drug Administration (R01FD003896). The authors thank the technical staff at SAFC Pharma for contract manufacture of study drug, the NHLBI Gene and Cell Therapy DSMB for review of clinical safety data, Ann Fu for histology services, and Theresa Hicks for decision monitoring services. We also thank Jeffrey Nickerson and Jean Underwood for their help with the confocal microscopy. Finally, we thank our study subjects for their interest and participation in this study.

Received for publication April 5, 2013, and accepted in revised form September 12, 2013.

Address correspondence to: Terence R. Flotte, University of Massachusetts Medical School Suite S1-340, 55 Lake Avenue North, Worcester, Massachusetts 01655, USA. Phone: 508.546.2107; Fax: 508.856.8181; E-mail: terry.flotte@umassmed.edu.

1. Maguire AM, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med*. 2008;358(21):2240–2248.
2. Hauswirth WW, et al. Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. *Hum Gene Ther*. 2008;19(10):979–990.
3. Cideciyan AV, et al. Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc Natl Acad Sci U S A*. 2008;105(39):15112–15117.
4. Eberling JL, et al. Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology*. 2008;70(21):1980–1983.
5. Muramatsu S, et al. A phase I study of aromatic L-amino acid decarboxylase gene therapy for Parkinson's disease. *Mol Ther*. 2010;18(9):1731–1735.
6. Nathwani AC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*. 2011;365(25):2357–2365.
7. LeWitt PA, et al. AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial. *Lancet Neurol*. 2011;10(4):309–319.
8. Stroes ES, et al. Intramuscular administration of AAV1-lipoprotein lipase S447X lowers triglycerides in lipoprotein lipase-deficient patients. *Arterioscler Thromb Vasc Biol*. 2008;28(12):2303–2304.
9. Mingozzi F, et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med*. 2007;13(4):419–422.
10. Pien GC, et al. Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *J Clin Invest*. 2009;119(6):1688–1695.
11. Flotte TR, et al. Phase I trial of intramuscular injection of a recombinant adeno-associated virus alpha 1-antitrypsin (rAAV2-CB-hAAT) gene vec-

tor to AAT-deficient adults. *Hum Gene Ther*. 2004; 15(1):93–128.
12. Brantly ML, et al. Phase I trial of intramuscular injection of a recombinant adeno-associated virus serotype 2 alphal-antitrypsin (AAT) vector in AAT-deficient adults. *Hum Gene Ther*. 2006;17(12):1177–1186.
13. Brantly ML, et al. Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. *Proc Natl Acad Sci U S A*. 2009;106(38):16363–16368.
14. Flotte TR, et al. Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing α1-antitrypsin: interim results. *Hum Gene Ther*. 2011;22(10):1239–1247.
15. Ye GJ, Oshins RA, Rouhani FN, Brantly ML, Chulay JD. Development, validation and use of ELISA for antibodies to human α-1 antitrypsin. *J Immunol Methods*. 2013;388(1–2):18–24.
16. Endl J, et al. Coexpression of CD25 and OX40 (CD134) receptors delineates autoreactive T-cells in type 1 diabetes. *Diabetes*. 2006;55(1):50–60.
17. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677–704.
18. Barber DL, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439(7077):682–687.
19. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell*. 2009;138(1):30–50.
20. Kaufmann DE, Walker BD. PD-1 and CTLA-4 inhibitory cosignaling pathways in HIV infection and the potential for therapeutic intervention. *J Immunol*. 2009;182(10):5891–5897.
21. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J Exp Med*. 2006; 203(10):2223–2227.
22. Rodino-Klapac LR, et al. A translational approach for limb vascular delivery of the micro-dystrophin gene without high volume or high pressure for treatment of Duchenne muscular dystrophy. *J Transl Med*. 2007;5:45.
23. Toromanoff A, et al. Safety and efficacy of regional intravenous (r.i.) versus intramuscular (i.m.) delivery of rAAV1 and rAAV8 to nonhuman primate skeletal muscle. *Mol Ther*. 2008;16(7):1291–1299.
24. Arruda VR, et al. Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B. *Blood*. 2010; 115(23):4678–4688.
25. Stieger K, et al. Detection of intact rAAV particles up to 6 years after successful gene transfer in the retina of dogs and primates. *Mol Ther*. 2009; 17(3):516–523.
26. Jordan MS, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2(4):301–306.
27. Sakaguchi S. Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. *J Exp Med*. 2003;197(4):397–401.
28. Larkin J, Picca CC, Caton AJ. Activation of CD4+ CD25+ regulatory T cell suppressor function by analogs of the selecting peptide. *Eur J Immunol*. 2007; 37(1):139–146.
29. Li S, et al. Analysis of FOXP3+ regulatory T cells that display apparent viral antigen specificity during chronic hepatitis C virus infection. *PLoS Pathog*. 2009;5(12):e1000707.
30. Ebinuma H, et al. Identification and in vitro expansion of functional antigen-specific CD25+ FoxP3+ regulatory T cells in hepatitis C virus infection. *J Virol*. 2008;82(10):5043–5053.
31. MacDonald AJ, et al. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J Infect Dis*. 2002;185(6):720–727.
32. Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonneil C, Levy Y. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*. 2004; 104(10):3249–3256.
33. van der Burg SH, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. *Proc Natl Acad Sci U S A*. 2007;104(29):12087–12092.
34. Mezzina M, Merten OW. Adeno-associated viruses. *Methods Mol Biol*. 2011;737:211–234.
35. Fan Z, et al. Safety and feasibility of high-pressure transvenous limb perfusion with 0.9% saline in human muscular dystrophy. *Mol Ther*. 2012; 20(2):456–461.
36. Toromanoff A, et al. Lack of immunotoxicity after regional intravenous (RI) delivery of rAAV to nonhuman primate skeletal muscle. *Mol Ther*. 2010; 18(1):151–160.
37. Chulay JD, et al. Preclinical evaluation of a recombinant adeno-associated virus vector expressing human α-1 antitrypsin made using a recombinant herpes simplex virus production method. *Hum Gene Ther*. 2011;22(2):155–165.
38. Sehouli J, et al. Epigenetic quantification of tumor-infiltrating T-lymphocytes. *Epigenetics*. 2011; 6(2):236–246.
39. Emerson R, Sherwood A, Desmarais C, Malhotra S, Phippard D, Robins H. Estimating the ratio of CD4+ to CD8+ T cells using high-throughput sequence data. *J Immunol Methods*. 2013;391(1–2):14–21.
40. Robins HS, et al. Comprehensive assessment of T-cell receptor β-chain diversity in αβ T cells. *Blood*. 2009;114(19):4099–4107.