5 Year Expression and Neutrophil Defect Repair after Gene Therapy in Alpha-1 Antitrypsin Deficiency

Christian Mueller,[1] Gwladys Gernoux,[1] Alisha M. Gruntman,[1,2] Florie Borel,[1] Emer P. Reeves,[3] Roberto Calcedo,[4] Farshid N. Rouhani,[5] Anthony Yachnis,[5] Margaret Humphries,[1] Martha Campbell-Thompson,[5] Louis Messina,[1] Jeffrey D. Chulay,[6] Bruce Trapnell,[7] James M. Wilson,[4] Noel G. McElvaney,[3] and Terence R. Flotte[1]

[1]University of Massachusetts Medical School, Worcester, MA 01655, USA; [2]Tufts University Cummings School of Veterinary Medicine, North Grafton, MA 01536, USA; [3]Department of Respiratory Research, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 2, Ireland; [4]Gene Therapy Program, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA; [5]University of Florida College of Medicine, Gainesville, FL 32610, USA; [6]Applied Genetic Technologies Corporation, Alachua, FL 32615, USA; [7]Cincinnatti Children's Hospital, Cincinnati, OH 45229, USA

Alpha-1 antitrypsin deficiency is a monogenic disorder resulting in emphysema due principally to the unopposed effects of neutrophil elastase. We previously reported achieving plasma wild-type alpha-1 antitrypsin concentrations at 2.5%–3.8% of the purported therapeutic level at 1 year after a single intramuscular administration of recombinant adeno-associated virus serotype 1 alpha-1 antitrypsin vector in alpha-1 antitrypsin deficient patients. We analyzed blood and muscle for alpha-1 antitrypsin expression and immune cell response. We also assayed previously reported markers of neutrophil function known to be altered in alpha-1 antitrypsin deficient patients. Here, we report sustained expression at 2.0%–2.5% of the target level from years 1–5 in these same patients without any additional recombinant adeno-associated virus serotype-1 alpha-1 antitrypsin vector administration. In addition, we observed partial correction of disease-associated neutrophil defects, including neutrophil elastase inhibition, markers of degranulation, and membrane-bound anti-neutrophil antibodies. There was also evidence of an active T regulatory cell response (similar to the 1 year data) and an exhausted cytotoxic T cell response to adeno-associated virus serotype-1 capsid. These findings suggest that muscle-based alpha-1 antitrypsin gene replacement is tolerogenic and that stable levels of M-AAT may exert beneficial neutrophil effects at lower concentrations than previously anticipated.

INTRODUCTION

Alpha-1 antitrypsin (AAT) deficiency is among the most prevalent monogenic disorders worldwide, with an estimated 181,000 individuals possessing the most common genotype associated with severe disease (homozygous E342K or PI*ZZ).[1] The common mutation leads to impaired secretion of AAT from its primary site of synthesis, the hepatocyte. The two primary diseases associated with AAT mutation are lung disease, due to a deficiency of AAT protein in circulation, and liver disease, which is due to the downstream consequences of the aggregation and polymerization of the mutant Z-AAT protein in hepatocytes.

The onset of respiratory disease in patients with AAT deficiency generally manifests in the fourth and fifth decade of life for smokers and can be delayed to the sixth decade for non-smokers, while some non-smoking AAT deficient patients never develop lung disease. The lung disease is thought to be due to absence or low activity of AAT and consequently its inability to fully inactivate neutrophil elastase and other neutrophil-derived proteases.[2] Over time, this anti-protease/protease imbalance leads to degradation of the alveolar walls and the surrounding connective tissue elastin matrix, which leads to a decrease in lung elasticity and surface area. The classic description includes panacinar emphysema, with a lower lobe predominance, but patients often manifest other symptoms, including cough and wheezing, which may relate to both an increase in airway inflammation and a decrease in airway tethering as a consequence of the loss of interstitial elastin.[3] Current therapy for AAT lung disease consists of weekly intravenous infusions of wild-type (M) AAT protein, along with supportive and compensatory therapies, such as antibiotics, oxygen supplementation, and lung transplantation for end-stage pulmonary disease.

Clinical gene augmentation therapy for AAT lung disease has been developed using recombinant viral or non-viral vectors that express M-AAT.[4–17] Cationic liposome delivery of AAT plasmid DNA to the respiratory epithelium was attempted to explore feasibility.[4–8] Intrapleural administration of rAAV-rh10-AAT has been explored more recently and is entering early phase trials.[12] Meanwhile, rAAV2-AAT and rAAV1-AAT vectors have been developed for
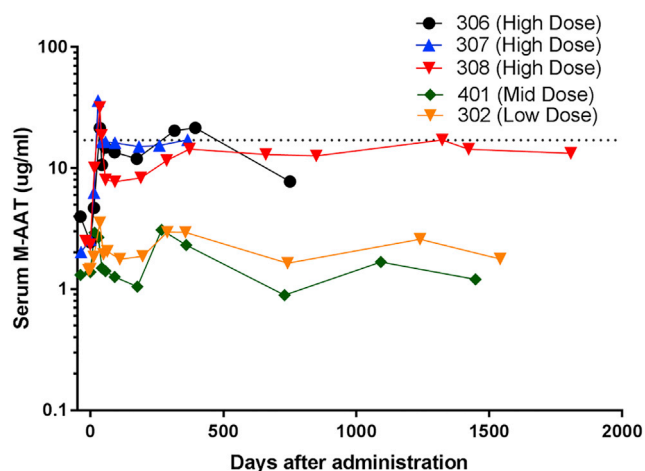
Figure 1. M-AAT Serum Levels over the 5 Years after Intramuscular Administration of rAAV1-CB-hAAT

The dotted line is at ~2.5% of the target therapeutic serum concentration of 572 μg/mL (11 μM). The serum AAT levels were assayed using a M-AAT-specific ELISA. The high dose was 6.0×10^{12} vg/kg, the mid dose was 1.9×10^{12} vg/kg, and the low dose was 6.0×10^{11} vg/kg. Early time points previously reported in Mueller et al.[18]

intramuscular (IM) administration by our group.[13–16,18,19] The rAAV1-AAT vector has been used in a phase 1 trial of IM administration in AAT deficient adults and has demonstrated dose-dependent, sustained expression of M-AAT in the absence of significant adverse effects.[13,16,18,19]

A phase 2a trial of direct IM injection of rAAV1-CB-hAAT was undertaken in nine adult patients with severe AAT deficiency, at doses ranging from 6.0×10^{11} vg/kg to 6.0×10^{12} vg/kg, in order to evaluate safety and to determine whether the dose response to vector would be linear within this range. The 3-month results of that trial indicated that vector administration was safe and that a linear dose-related increase in serum M-specific AAT levels was observed.[13] Interestingly, the vector used in this trial was produced using an HSV1-based helper system, as compared with a transfection-based system in the phase 1 trial, and the phase 2a trial confirmed an increased bioactivity of vector on a vg/kg basis. Longer-term follow up of the subjects in this trial at 12–18 months after vector administration demonstrated that vector expression persisted at high levels despite an ongoing AAV1-capsid specific ELISPOT response in peripheral blood, and this persistence was linked to the presence of capsid-specific regulatory T cells (Tregs) in the muscle and the peripheral blood and to PD-1 expression in the muscle.[18] The current report extends these findings with an approximately 5 year follow up of vector expression, anti-protease biomarkers, and immune profiles. We primarily focused on one patient (#308) from the original study who has not been treated with AAT protein replacement therapy. These studies demonstrated continued stable transgene expression at 5 years after administration and examined the effects of sustained M-AAT protein expres-

sion on neutrophil defects that have been associated with the AAT deficiency state and immunologic parameters in blood and muscle. In addition, we demonstrate two of the three patients in the high dose group also have increased serum anti-elastase activity after gene therapy.

RESULTS

Stable Transgene Expression from Muscle

In order to determine the stability of transgene expression after one-time IM administration of rAAV1-hAAT, serum M-AAT levels were determined at yearly intervals from subjects in a previously published phase 2a trial who were not receiving AAT augmentation therapy. Subject 308, in the high dose cohort, had stable transgene expression at a level greater than 2% of the accepted therapeutic target of 11 μM (Figure 1) as determined by a previously published M-AAT specific ELISA.[16] A muscle biopsy from this patient, performed at 5 years after vector dosing, also showed ongoing transgene expression, as indicated by substantial AAT immunoreactivity within the sarcoplasm of myofibers (Figure 2). Myofibers varied substantially in the intensity of AAT staining, with scattered high-expressing fibers interspersed among fibers with little or no detectable reactivity. A summary of the hAAT immunohistochemistry results are provided in Table 1.

Histological Features of the Injection Site

The histological features of muscle at the injection site were examined by hematoxylin-eosin staining and semiquantitative immunophenotyping (Figure 3). The overall profile of cellular infiltrates is described in Table 2. Immunohistochemical staining indicated rare "microfoci" of CD3+ inflammatory infiltrates. CD3-immunoreactive T-lymphocytes comprised the most abundant single subset of mononuclear cells, with CD8+ cells accounting for slightly more of the total inflammatory cell population than CD4-reactive cells. Scattered CD20+ B-lymphocytes and CD68+ macrophages were also seen. However, when compared with the results reported from the 3- and 12-month biopsies from the same patient, the abundance of infiltrating lymphocytes of all types appears to have decreased. Bisulfite sequencing was used to quantitatively characterize the immunophenotyping results and examine the unique methylation status of the activated FoxP3 promoter (indicative of FoxP3+ T_{reg} cells), as well as the methylations signature of the activated CD8 locus associated with CD8-expressing cells (Figure 4). The absolute sequencing counts for both the CD8+ and T_{regs} decreases over time as was observed in the histological evaluation. However, as reported at the 12-month time point,[18] a relatively high proportion of T_{regs} is observed and maintained over time.

Characterization of T Cells from the Injected Muscle

In order to study in situ infiltrating lymphocytes more thoroughly, cells were extracted from the muscle biopsy tissue using previously published methods and analyzed by flow cytometry (Figure 5).[20] These analyses confirmed the bisulfite sequencing data and also
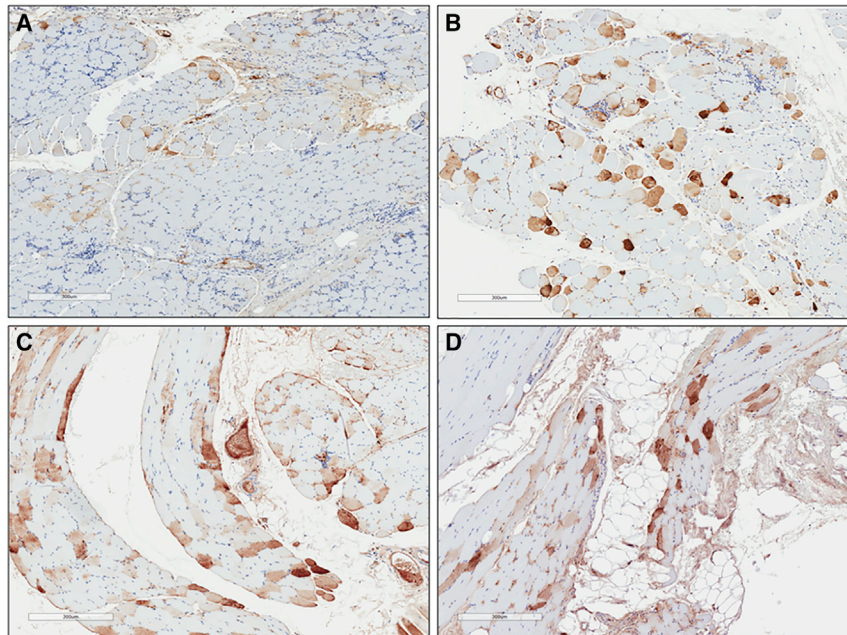
Figure 2. M-AAT Expression in Skeletal Muscle 3 Months, 12 Months, and 5 Years after Intramuscular Administration of rAAV1-CB-hAAT

Muscle immunochemistry staining for hAAT. The specimens were biopsied from the area injected with rAAV1 and the tissue stained for hAAT (brown). The AAT expression was persistent, while inflammatory cell infiltrates decreased over time from 3 months to 5 years. All of the slides are from patient 308. (A) Tissue 3 months after rAAV1-CB-hAAT delivery. (B) Tissue 12 months after rAAV1-CB-hAAT delivery. (C and D) Tissue 5 years after rAAV1-CB-hAAT delivery.

showed the presence of T_{regs} among $CD3^+CD4^+$ muscle-infiltrating lymphocytes (MIL), with greater than 6% of $CD4^+$ cells demonstrating the $Helios^+FoxP3^+$ T_{reg} phenotype. Further analysis of the status of the muscle infiltrating T_{regs} revealed that more than 75% of the cells were activated as assessed by the upregulation of both CD25 and OX-40. In contrast to the MIL T_{reg} population, which was characterized by general activated status, about 10% of the $CD8^+$ MIL population was expressing high levels of PD-1 and LAG-3, consistent with T cell exhaustion.

Characterization of Peripheral Blood T Cell Responses to AAV1 Capsid Epitopes

Specific peripheral blood T cell responses to AAV1 capsid epitopes were examined using a gamma-interferon ELISPOT assay. Peripheral blood reactivity to AAV1 epitopes, when compared with that observed at earlier time points, had decreased in magnitude,

Table 1. Summary of hAAT Immunohistochemistry Results at the Indicated Time Points in Muscle Biopsies from Subject 308

Time Point	308 3 Months		308 12 Months		308 5 Years
Sample number	1	2	1	2	1
Myofibers	1+	1+	NE	1+	1+
Endomysium	1+	1+	NE	1+	1+
Intravascular	3+	3+	NE	3+	3+
Vascular endothelium	NE	NE	NE	3+	2+

Scoring indicates positive hAAT staining in different regions of the muscle sample. hAAT expression was maintained through the 5 year time point. The scoring scale is as follows: 0 (absent, none), 1+ (occasional, focal, mild, scattered, and slight), 2+ (prominent, marked, and moderate), or 3+ (strong, extensive, and severe). NE = not examined.

but remained significantly above background within epitope pools AAV1b and AAV1c at 52 months after injection (Figure 6).

Biomarkers of Biological Activity of Transgene-Expressed AAT

The augmentation of PiZ homozygous individuals with stable levels of M-AAT between 2% and 3% of the therapeutic threshold has never been studied with protein replacement or by any other means. The following observations therefore constitute the first description of the functional effect of augmenting M-AAT below the canonical therapeutic threshold of 11 μM (572 μg/mL). The most direct functional assay for AAT bioactivity is to quantify its ability to inactivate neutrophil elastase (NE). Given that secreted AAT from AAV transduced muscle reaches steady-state levels in serum, we analyzed the ability of the patients' sera to inactivate human elastase otherwise known as the sera's anti-neutrophil elastase capacity (ANEC). As shown in Figure 7, two out of three patients had low ANEC activity prior to the gene therapy as compared to a cohort of control PiM individuals. ANEC activity for patient 308 was, however, high and comparable to that of a PiM (wild-type) patient. This was not accounted for by total AAT or PiZ AAT levels, suggesting that this individual had an elevated level of another serum anti-protease, which may have accounted for her high ANEC and her mild clinical phenotype. At 3 months post-gene delivery, the ANEC activity was strongly increased in patient 306 and patient 307, which is highly suggestive of a biological effect in these two subjects; it remained stable in patient 308. At 1 year post intervention, the ANEC activity remained either comparable to the 3 month values (patients 306 and 308) or increased further (patient 307). Patients 306 and 307 subsequently started protein augmentation therapy and their ANEC activity further increased to normal levels at the 5 year time point. Patient 308, who remained off protein augmentation therapy 5 years after gene delivery, maintained stable yet high ANEC activity.

Surface anti-lactoferrin staining on neutrophils and circulating serum tumor necrosis factor receptor 1 (TNF-R1) have been recently demonstrated to respond to augmentation therapy.[21] Both these
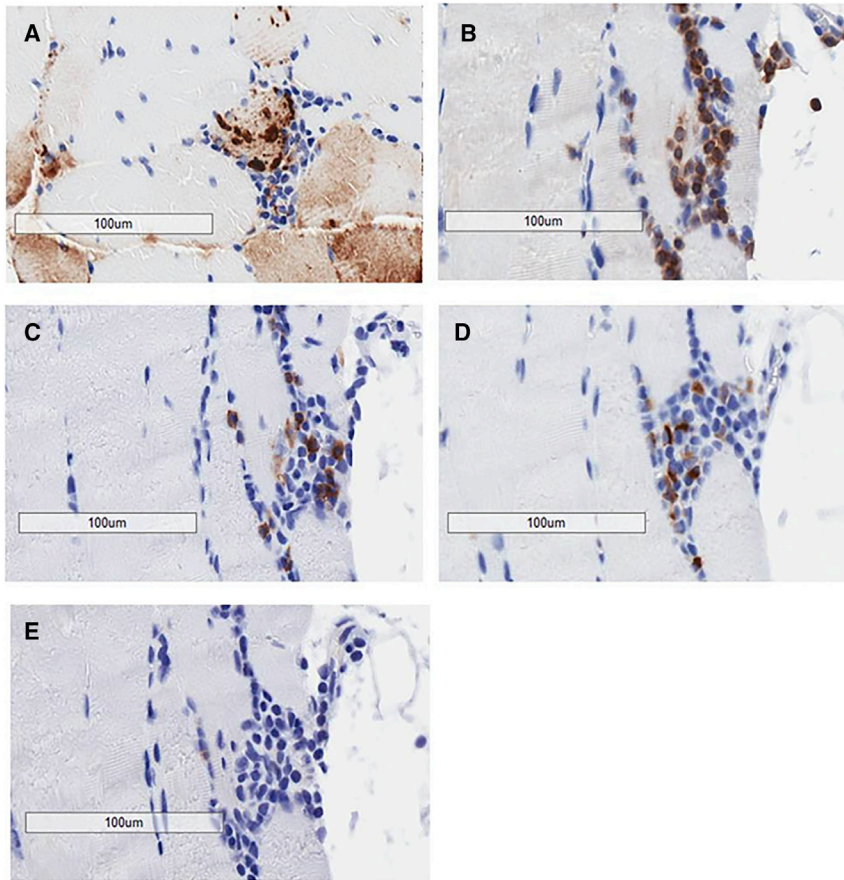
Figure 3. Immunophenotyping of Cellular Infiltrates in Skeletal Muscle at the Injection Site 5 Years after Intramuscular Administration of rAAV1-CB-hAAT

(A) hAAT immunohistochemistry showing microfoci of immune cells around cell expression hAAT. (B) CD3 staining, demonstrating that many of the mononuclear immune cells are T-lymphocytes. (C) CD8 staining indicating a population of cytotoxic T cells. (D) CD68 staining indicating a population of macrophages. (E) CD20 staining indicating a small population of B-lymphocytes.

the worst clinical condition of any patient in the high-dose cohort and required a lung transplant and was subsequently diagnosed with pulmonary Aspergillosis, prior to collection of the 5 year serum.

DISCUSSION

The multiyear persistence of rAAV expression in human gene therapy trial participants has been reported after a number of routes of delivery, including subretinal, intravenous, and intramuscular routes. However, this is the first report in which such results were obtained without immune suppression or corticosteroid therapy. The fact that this persistence in the absence of immune suppression coincides with ongoing evidence of an AAV1-capsid specific T_{reg} response and of CD8$^+$ T cell exhaustion suggests that these mechanisms may be operative in this context. It should be noted that in an earlier report from this trial at the 1 year follow up time point, we could not identify cells positive for Treg markers in normal control muscle tissue, further supporting that this finding is unique to the vector injected muscle.[18] While the T_{reg} percentage in the vector-injected muscle was approximately 10% of all CD3+ T cells, it should be noted that biological activity of T_{reg} cells in functional assays is typically observed at ratios as low as 0.1%.[22] What remains unclear is whether these immunologic findings are generalizable to intramuscular gene therapy with rAAV expressing other transgenes and at other doses, or indeed whether this might be observed with other routes of delivery. It is also not clear whether the levels observed in the current study could have been further enhanced with corticosteroid or other immune suppressive therapy.

The finding of partial correction of multiple indicators of AAT biological activity, including elastase inhibition, auto-antibodies, and the neutrophil degranulation phenotype in AAT deficient patients is also quite significant. The target level of 11 μM (570 mcg/mL) that has been used as the target for protein and gene replacement was based on compound heterozygotes with the genotype PI*SZ. Since neither the S nor the Z variants of AAT have full specific activity, it is unknown precisely what level of M-AAT would be

markers were found to decrease significantly after gene therapy (Figure 8). Remarkably, two out three patients in high dose cohort showed approximately %50 correction of the circulating TNF-R1, suggesting that significant biological activity may have been achieved (Figure 8A). It should be noted that patient 306 did not show a decrease at the 5 year time point. This patient had

Table 2. Summary of Leukocyte Phenotyping Immunohistochemistry Results at the Indicated Time Points in Muscle Biopsies from Subject 308

Subject Number	308 3 Months		308 12 Months		308 5 Years
Sample number	1 (%)	2 (%)	1	2 (%)	1 (%)
CD3	2+50	3+70	NE	3+75	1+5
CD4	1+20	2+25	NE	2+30	1+5
CD8	2+30	2+45	NE	2+45	1+5
CD20	1+20	1+15	NE	1+5	1+5
CD68	2+30	1+10	NE	1+20	1+5

Inflammatory scores decreased over time following vector injection including an overall decrease in the percentage of all inflammatory cell types. CD3: staining for T-lymphocytes. CD4: staining for help T cells. CD8: staining for cytotoxic T cells. CD20: staining for B-lymphocytes. CD68: staining for macrophages. Phenotype results were scored 1+ (<25%), 2+ (25%–65%), or 3+ (>65%).
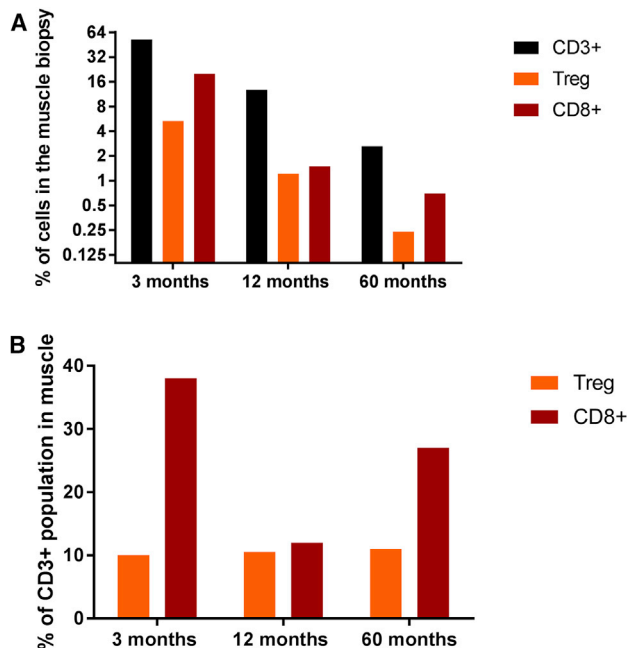
Figure 4. Bisulfite Sequencing to Quantify the Percentage of Immune Cells in Muscle Biopsies Obtained 5 Years after Intramuscular Administration of rAAV1-CB-hAAT

A relatively high proportion of T_{regs} was observed in the muscle immune cell population. (A) Percentage of $CD3^+$, T_{reg} ($FOXP3^+$), and $CD8^+$ cells in the muscle. (B) T_{reg} and $CD8^+$ cells as a percentage of $CD3^+$ cell population in the muscle.

required to achieve an equivalent biological effect. The IV protein replacement of M-AAT, which is the current standard of therapy, is not an ideal surrogate for establishing a target for gene therapy, because of the drastic peaks and troughs of serum levels observed after each weekly or bi-weekly infusion. In fact, there is no clinical precedent for what was achieved in the patients described here, where approximately 2.5% of the target level was produced continuously, without any peak and trough effects. Theoretically, this could exert a greater pharmacodynamic effect, analogous to the greater benefit afforded by insulin pumps in patients with diabetes in prevention of microvascular complications, as compared with the same total daily insulin dose when administered in split doses. Thus, the findings of biological efficacy in this context could be of major significance if they can be confirmed in future studies and are later found to correlate with clinically meaningful effects.

Earlier reports from this trial indicated a reproducible dose-response relationship between the dose of rAAV1-AAT and the serum levels of M-AAT observed.[13] This bodes well for future studies in which alternative methods of muscle delivery, such as limb perfusion, might enable a further increase in dose and in the absolute serum M-AAT level achieved. Other routes of delivery, such as intravenous or intrapleural, are also being investigated.[12] Intravenous delivery, similar to that used in the hemophilia B and

hemophilia A trials, would almost certainly require the use of corticosteroids or other immune suppressive drugs. There is also a theoretical concern that overexpressing M-AAT within hepatocytes of PiZ patients could exacerbate subclinical liver disease in these patients, most of whom show histologic evidence of a substantial load of retained mutant within their hepatocytes.[23,24] Thus, given the encouraging findings regarding the sustained biologic activity and immunologic tolerance seen with IM delivery, it would seem worthwhile to continue to pursue the muscle-based platform for gene replacement of this and possibly other secreted proteins. In addition to modifying the delivery method for this vector, future studies could theoretically further enhance the M-AAT expression by modifications to the vector capsid, promoter, and through codon optimization.

MATERIALS AND METHODS

Experimental Design

As indicated in previous reports, the study was conducted as an open-label, non-randomized, multicenter, sequential, 3-arm, phase II clinical trial to evaluate the safety and efficacy of administering a rAAV1-CB-hAAT vector intramuscularly.[13,18] The study was conducted under an IND with approval of the 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 with Clinical Trials.gov (NCT01054339). There were nine subjects (three per cohort) that received intramuscular 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.[13]

Laboratory Assessments, In Situ Staining, and Bisulfite Sequencing

AAT serum levels and ELISPOTs were done as previously described in the 3 month interim report.[13] Immunohistochemical staining to detect hAAT protein within myofibers as well as to characterize cell surface markers on infiltrating cells was performed as previously reported.[18] Bisulfite sequencing of immune cells was done as previously described in the 1 year report.[18]

Regulatory T Cell and Exhausted T Cell Detection by Flow Cytometry

The patient muscle biopsy sample was cut into approximately 5 mm³ pieces and incubated in RPMI media supplemented with DNase I (62.5 U/mL, Zymo Research) and collagenase (0.25 mg/mL, Roche) for 2 hr at 37°C. Cell suspension was homogenized and filtered using a 70 μm cell strainer before staining. Regulatory T cells and exhausted $CD8^+$ T cell populations were analyzed using CD3/CD4/Helios/FoxP3/CD25/OX40 and CD3/CD8/PD1/LAG3 markers, respectively. Extracellular staining consisted of cell incubation on ice for 30 min. Cells were then washed twice with $1\times$ PBS and resuspended in $1\times$ PBS supplemented with 0.5% FBS and 2 mM EDTA. FoxP3 and Helios intracellular staining was performed according to manufacturer's recommendations (anti-human FoxP3 Flow Kit,
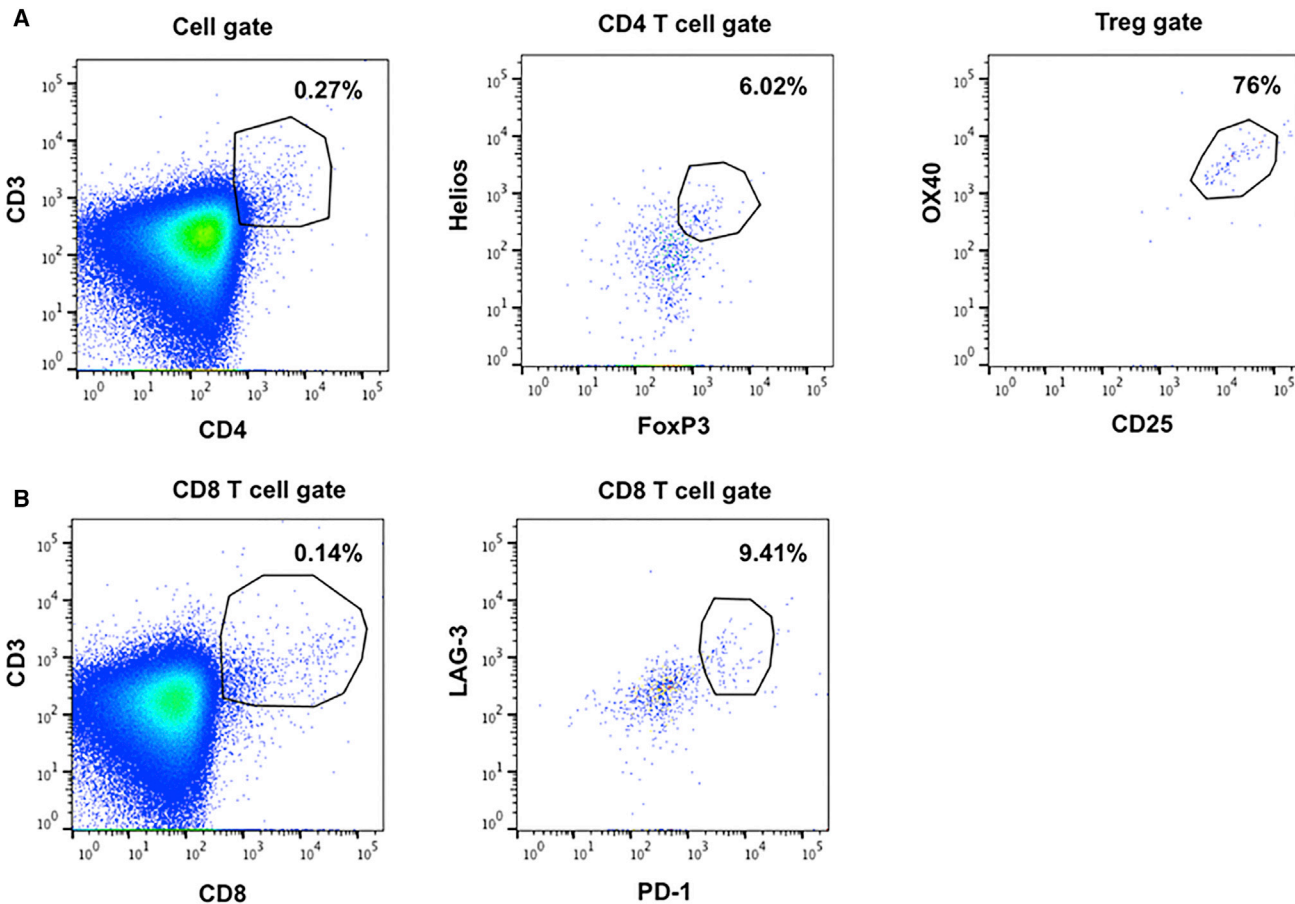
Figure 5. Regulatory T Cell and Exhausted T Cell Detection by Flow Cytometry in Samples from Muscle Biopsies Obtained 5 Years after Intramuscular Administration of rAAV1-CB-hAAT

(A) Regulatory T cells were gated as Helios+FoxP3+ cells in the CD3+CD4+ subset. The activated T_{reg} cells were defined as OX40+CD25+ cells. (B) Exhausted CD8 T cells were gated as LAG-3+PD-1+ cells in the CD3+CD8+ subset. The percentages of each population are indicated in the dot plots.

BioLegend). Cells were acquired using a FACS-LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (version 10.1; Tree Star).

Neutrophil Elastase Activity

Relative serum neutrophil elastase activity was quantified by a colorimetric assay, according to the manufacturer's instructions (Enzo Life
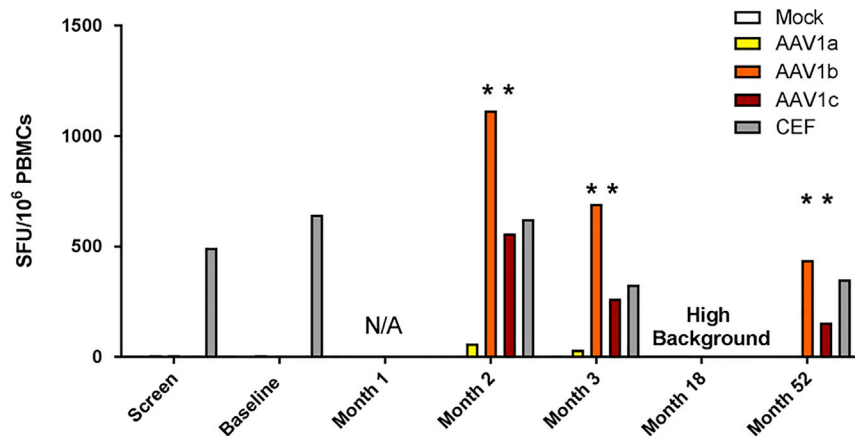
Figure 6. Peripheral Gamma-IFN ELISPOT Responses to AAV Capsid Epitope Pools

A decreased peripheral blood AAV1 reactivity was observed at month 52 compared to earlier time points (all of the data from patient 308). SFU, spot forming units; PBMC, peripheral blood mononuclear cell; AAV1a/b/c, pools of AAV1 peptides; CEF, positive control peptide pool.
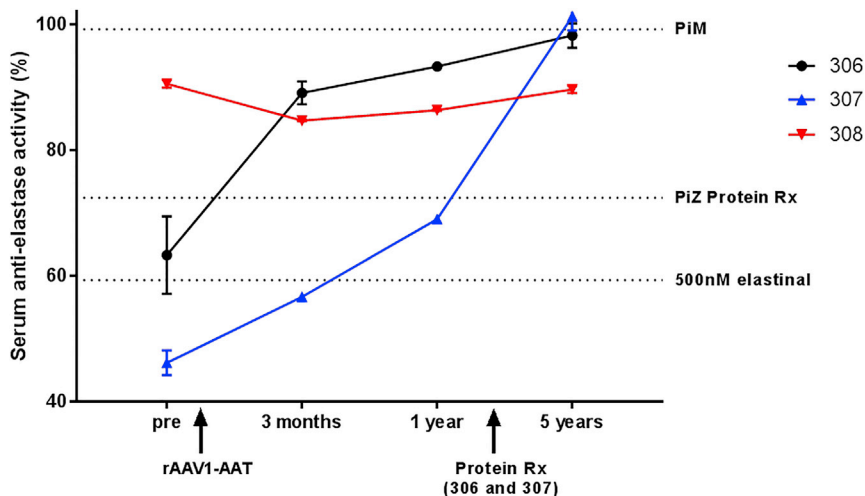
Figure 7. Elastase Inhibitory Activity Assay

Inhibition of neutrophil elastase was quantified by a colorimetric assay in triplicates and reported as % of no inhibitor control ±SEM. The patients were dosed with rAAV1-AAT gene therapy treatment at day 0 and serum was sampled pretreatment and at 3 months, 1 year, and 5 years post treatment. Patients 306 and 307 started protein augmentation therapy (Protein Rx) after their 1 year follow up. Additional controls included two PiM (MM) individuals and two PiZ (ZZ) individuals under protein augmentation therapy (PiZ Protein Rx) (500 nM Elastinal indicates the elastase activity control).

Sciences). Briefly, neutrophil elastase was diluted in 37°C assay buffer to 2.2 μU/μL. Elastinal (100 μM) or patient serum was added to each well followed by a 30 min incubation at 37°C to allow inhibitor/elastase interaction. Substrate was subsequently added (100 μM) and absorbance A_{450nm} was read every minute for 60 min. Data were analyzed by determining the slope of the kinetic for each sample, the slopes of the three technical replicates were averaged, values were reduced by subtracting the slope of the blank (buffer and substrate), and normalized by setting the control (buffer, substrate, and elastase) at 100%.

Neutrophil Anti-Lactoferrin and TNF-R1 Assay

Fixed and blocked neutrophils (1×10^6/mL in PBS) were incubated with Rb anti-lactoferrin primary (2 mg) antibody or isotype control antibody (Rb IgG) for 2 hr at room temperature.

After two steps of washing in PBS, cells were then incubated with Gt-anti-rabbit FITC labeled secondary antibody (1 hr at room temperature) and then washed again. Fluorescence was counted using a BD FACSCalibur flow cytometer (BD Bioscience) with a total of 10,000 events acquired. The data were analyzed using BD CellQuest

Pro software. The TNF-R1 assay was performed as previously described.[21]
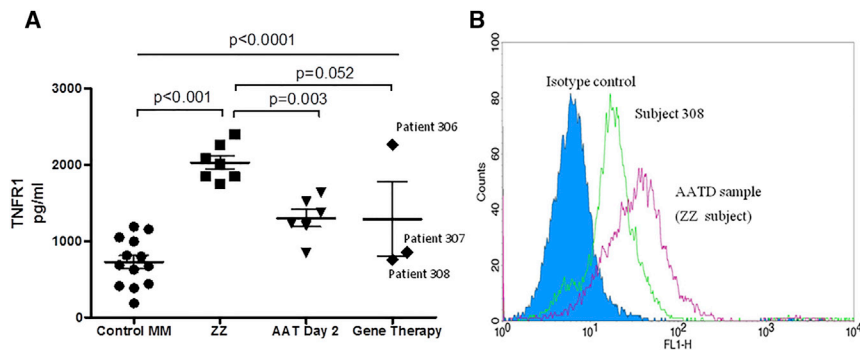
AUTHOR CONTRIBUTIONS

Figure 8. Biomarkers of Degranulation and Auto-Antibodies: TNF-R1 and Neutrophil Anti-Lactoferrin

TNF-R1 levels in patients treated with gene therapy approached that of control PiM patients (M-AAT/MM). Anti-lactoferrin staining indicated that the rAAV1-AAT treated patient had an approximately 50% correction in this marker. (A) TNFR1 levels in the serum 5 years post-gene delivery. The patient 306 serum data were collected following lung transplant and subsequent pulmonary Aspergillosis diagnosis. (B) Anti-lactoferrin levels in PFA fixed neutrophils (AAT Day 2: Day 2 after protein replacement therapy in a PiZ [ZZ] patient).

REFERENCES

1. de Serres, F., and Blanco, I. (2014). Role of alpha-1 antitrypsin in human health and disease. J. Intern. Med. *276*, 311–335.

2. Larsson, C. (1978). Natural history and life expectancy in severe alpha1-antitrypsin deficiency, Pi Z. Acta Med. Scand. *204*, 345–351.

3. Browne, R.J., Mannino, D.M., and Khoury, M.J. (1996). Alpha 1-antitrypsin deficiency deaths in the United States from 1979-1991. An analysis using multiple-cause mortality data. Chest *110*, 78–83.

4. Aliño, S.F., Crespo, J., Bobadilla, M., Lejarreta, M., Blaya, C., and Crespo, A. (1994). Expression of human alpha 1-antitrypsin in mouse after in vivo gene transfer to hepatocytes by small liposomes. Biochem. Biophys. Res. Commun. *204*, 1023–1030.

5. Aliño, S.F., Bobadilla, M., Crespo, J., and Lejarreta, M. (1996). Human alpha 1-antitrypsin gene transfer to in vivo mouse hepatocytes. Hum. Gene Ther. *7*, 531–536.

6. Crepso, J., Blaya, C., Crespo, A., and Aliño, S.F. (1996). Long-term expression of the human alpha1-antitrypsin gene in mice employing anionic and cationic liposome vectors. Biochem. Pharmacol. *51*, 1309–1314.

7. Aliño, S.F. (1997). Long-term expression of the human alpha1-antitrypsin gene in mice employing anionic and cationic liposome vector. Biochem. Pharmacol. *54*, 9–13.

8. Canonico, A.E., Conary, J.T., Meyrick, B.O., and Brigham, K.L. (1994). Aerosol and intravenous transfection of human alpha 1-antitrypsin gene to lungs of rabbits. Am. J. Respir. Cell Mol. Biol. *10*, 24–29.

9. Brigham, K.L., Lane, K.B., Meyrick, B., Stecenko, A.A., Strack, S., Cannon, D.R., Caudill, M., and Canonico, A.E. (2000). Transfection of nasal mucosa with a normal alpha1-antitrypsin gene in alpha1-antitrypsin-deficient subjects: comparison with protein therapy. Hum. Gene Ther. *11*, 1023–1032.

10. De, B., Heguy, A., Leopold, P.L., Wasif, N., Korst, R.J., Hackett, N.R., and Crystal, R.G. (2004). Intrapleural administration of a serotype 5 adeno-associated virus coding for alpha1-antitrypsin mediates persistent, high lung and serum levels of alpha1-antitrypsin. Mol. Ther. *10*, 1003–1010.

11. Siegfried, W., Rosenfeld, M., Stier, L., Stratford-Perricaudet, L., Perricaudet, M., Pavirani, A., Lecocq, J.P., and Crystal, R.G. (1995). Polarity of secretion of alpha 1-antitrypsin by human respiratory epithelial cells after adenoviral transfer of a human alpha 1-antitrypsin cDNA. Am. J. Respir. Cell Mol. Biol. *12*, 379–384.

12. Chiuchiolo, M.J., Kaminsky, S.M., Sondhi, D., Hackett, N.R., Rosenberg, J.B., Frenk, E.Z., Hwang, Y., Van de Graaf, B.G., Hutt, J.A., Wang, G., et al. (2013). Intrapleural administration of an AAVrh.10 vector coding for human α1-antitrypsin for the treatment of α1-antitrypsin deficiency. Hum. Gene Ther. Clin. Dev. *24*, 161–173.

13. Flotte, T.R., Trapnell, B.C., Humphries, M., Carey, B., Calcedo, R., Rouhani, F., Campbell-Thompson, M., Yachnis, A.T., Sandhaus, R.A., McElvaney, N.G., et al. (2011). Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing α1-antitrypsin: interim results. Hum. Gene Ther. *22*, 1239–1247.

14. Song, S., Morgan, M., Ellis, T., Poirier, A., Chesnut, K., Wang, J., Brantly, M., Muzyczka, N., Byrne, B.J., Atkinson, M., and Flotte, T.R. (1998). Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. Proc. Natl. Acad. Sci. USA *95*, 14384–14388.

15. Song, S., Scott-Jorgensen, M., Wang, J., Poirier, A., Crawford, J., Campbell-Thompson, M., and Flotte, T.R. (2002). Intramuscular administration of recombinant adeno-associated virus 2 alpha-1 antitrypsin (rAAV-SERPINA1) vectors in a nonhuman primate model: safety and immunologic aspects. Mol. Ther. *6*, 329–335.

16. Brantly, M.L., Spencer, L.T., Humphries, M., Conlon, T.J., Spencer, C.T., Poirier, A., Garlington, W., Baker, D., Song, S., Berns, K.I., et al. (2006). Phase I trial of intramuscular injection of a recombinant adeno-associated virus serotype 2 alpha1-antitrypsin (AAT) vector in AAT-deficient adults. Hum. Gene Ther. *17*, 1177–1186.

17. Mueller, C., Tang, Q., Gruntman, A., Blomenkamp, K., Teckman, J., Song, L., Zamore, P.D., and Flotte, T.R. (2012). Sustained miRNA-mediated knockdown of mutant AAT with simultaneous augmentation of wild-type AAT has minimal effect on global liver miRNA profiles. Mol. Ther. *20*, 590–600.

18. Mueller, C., Chulay, J.D., Trapnell, B.C., Humphries, M., Carey, B., Sandhaus, R.A., McElvaney, N.G., Messina, L., Tang, Q., Rouhani, F.N., et al. (2013). Human Treg responses allow sustained recombinant adeno-associated virus-mediated transgene expression. J. Clin. Invest. *123*, 5310–5318.

19. Brantly, M.L., Chulay, J.D., Wang, L., Mueller, C., Humphries, M., Spencer, L.T., Rouhani, F., Conlon, T.J., Calcedo, R., Betts, M.R., et al. (2009). Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. Proc. Natl. Acad. Sci. USA *106*, 16363–16368.

20. Liang, F., Ploquin, A., Hernández, J.D., Fausther-Bovendo, H., Lindgren, G., Stanley, D., Martinez, A.S., Brenchley, J.M., Koup, R.A., Loré, K., and Sullivan, N.J. (2015). Dissociation of skeletal muscle for flow cytometric characterization of immune cells in macaques. J. Immunol. Methods *425*, 69–78.

21. Bergin, D.A., Reeves, E.P., Hurley, K., Wolfe, R., Jameel, R., Fitzgerald, S., and McElvaney, N.G. (2014). The circulating proteinase inhibitor α-1 antitrypsin regulates neutrophil degranulation and autoimmunity. Sci. Transl. Med. *6*, 217ra1.

22. Mahne, A.E., Klementowicz, J.E., Chou, A., Nguyen, V., and Tang, Q. (2015). Therapeutic regulatory T cells subvert effector T cell function in inflamed islets to halt autoimmune diabetes. J. Immunol. *194*, 3147–3155.

23. Lomas, D.A., Evans, D.L., Finch, J.T., and Carrell, R.W. (1992). The mechanism of Z alpha 1-antitrypsin accumulation in the liver. Nature *357*, 605–607.

24. Lomas, D.A. (1994). Loop-sheet polymerization: the structural basis of Z alpha 1-antitrypsin accumulation in the liver. Clin. Sci. *86*, 489–495.