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INTRODUCTION

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.

Alpha-1 antitrypsin 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 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.15 Meanwhile, rAAV2-AAT and rAAV1-AAT vectors have been developed for
intramuscular (IM) administration by our group. 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.

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 $6.0 \times 10^{12}$ vg/kg to $6.0 \times 10^{13}$ 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-AAT levels was observed. 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. 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 expression 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. 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 semi-quantitative 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 (inducive of FoxP3+ Treg cells), as well as the methylation signatures of the activated CD8 locus associated with CD8-expressing cells (Figure 4). The absolute sequencing counts for both the CD8+ and Treg decreases over time as was observed in the histological evaluation. However, as reported at the 12-month time point, a relatively high proportion of Treg 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). These analyses confirmed the bisulfite sequencing data and also
showed the presence of $T_{reg}$ among CD3+CD4+ muscle-infiltrating lymphocytes (MIL), with greater than 6% of CD4+ cells demonstrating the Helios+FoxP3+ Treg phenotype. Further analysis of the status of the muscle infiltrating $T_{reg}$ 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 Treg 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, 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. Both these
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 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 Treg 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 Treg percentage in the vector-injected muscle was approximately 10% of all CD3+ T cells, it should be noted that biological activity of Treg 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 PPSZ. 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.

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

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>308 3 Months</th>
<th>308 12 Months</th>
<th>308 5 Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample number</td>
<td>1 (%)</td>
<td>2 (%)</td>
<td>1 (%)</td>
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<td>CD3</td>
<td>2+50</td>
<td>3+70</td>
<td>NE</td>
</tr>
<tr>
<td>CD4</td>
<td>1+20</td>
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<td>CD68</td>
<td>2+30</td>
<td>1+10</td>
<td>NE</td>
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</tbody>
</table>

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 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.
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. 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. 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. 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. 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.

Laboratory Assessments, In Situ Staining, and Bisulfite Sequencing
AAT serum levels and ELISPOTs were done as previously described in the 3 month interim report. Immunohistochemical staining to detect hAAT protein within myofibers as well as to characterize cell surface markers on infiltrating cells was performed as previously reported. Bisulfite sequencing of immune cells was done as previously described in the 1 year report.

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× PBS and resuspended in 1× 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,
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 Sciences).

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**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 Treg 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.

**Figure 6. Peripheral Gamma-INF 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.
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 A450nm 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**
C.M. helped oversee and coordinate the study, designed and interpreted the immunology and ANEC experiments, and assisted with the writing of the manuscript; G.G. performed and designed the in situ immune and flow cytometry studies and helped interpret and write up those results; A.M.G. helped coordinate sample collection, isolated neutrophils for the anti-lactoferrin assay, and assisted with writing of the manuscript; F.B. performed the ANEC assays and helped interpret and write up their results; E.P.R. coordinated, ran, and helped interpret the TNF-R and anti-lactoferrin assays; R.C. ran and interpreted the ELISPOT data; F.N.R. performed M-AAT ELISA on patient serum; A.Y. interpreted and reported muscle histology results; M.H. coordinated patient sampling, processed the blood samples, and reviewed the manuscript; M.C.-T. coordinated and performed all muscle histology; L.M. performed all muscle biopsy sampling; B.T. dosed a patient in the original trial; J.D.C. aided in study design, interpretation, and manuscript revision; N.G.M. interpreted the TNF-R and

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**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).

**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 postgene 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).
anti-lactoferrin assays and aided with manuscript preparation; and T.R.F. designed the study, dosed the patients, interpreted the data, and wrote the manuscript.

CONFLICTS OF INTEREST
J.D.C. holds equity and stock options in Applied Genetic Technologies Corporation (AGTC). T.R.F. was a founder of AGTC, but donated his shares to the Alpha One Foundation, which has subsequently transferred them to AlphaNet. J.M.W. and T.R.F. are inventors on patents involving AAV that have been licensed to various biopharmaceutical companies. J.M.W. is a consultant to inventors on patents licensed to various biopharmaceutical companies. J.M.W. is a grantee from affiliates of ReGenX Holdings. In addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.

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