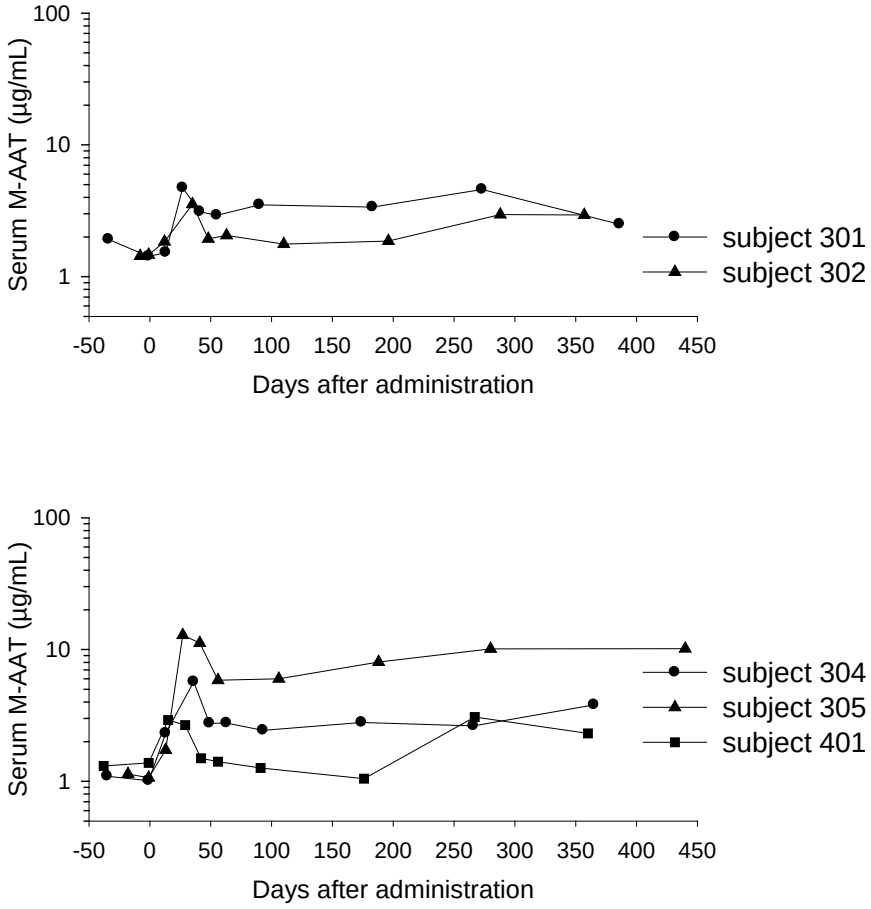
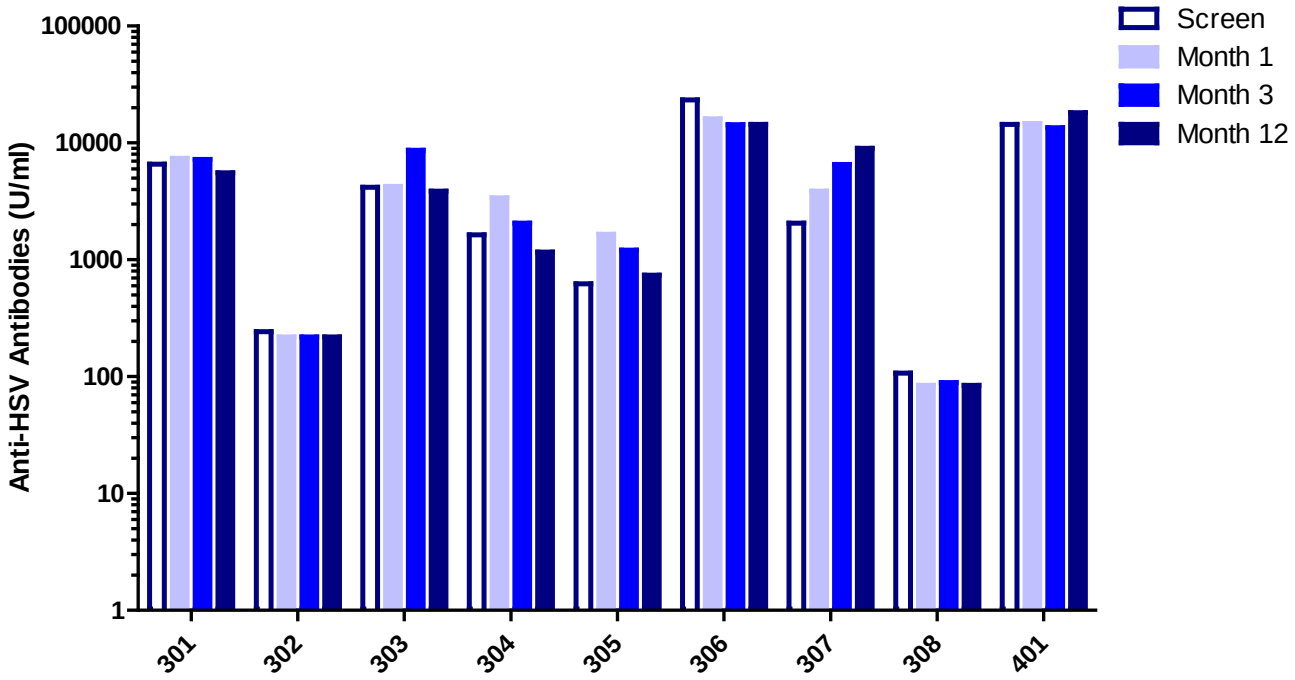
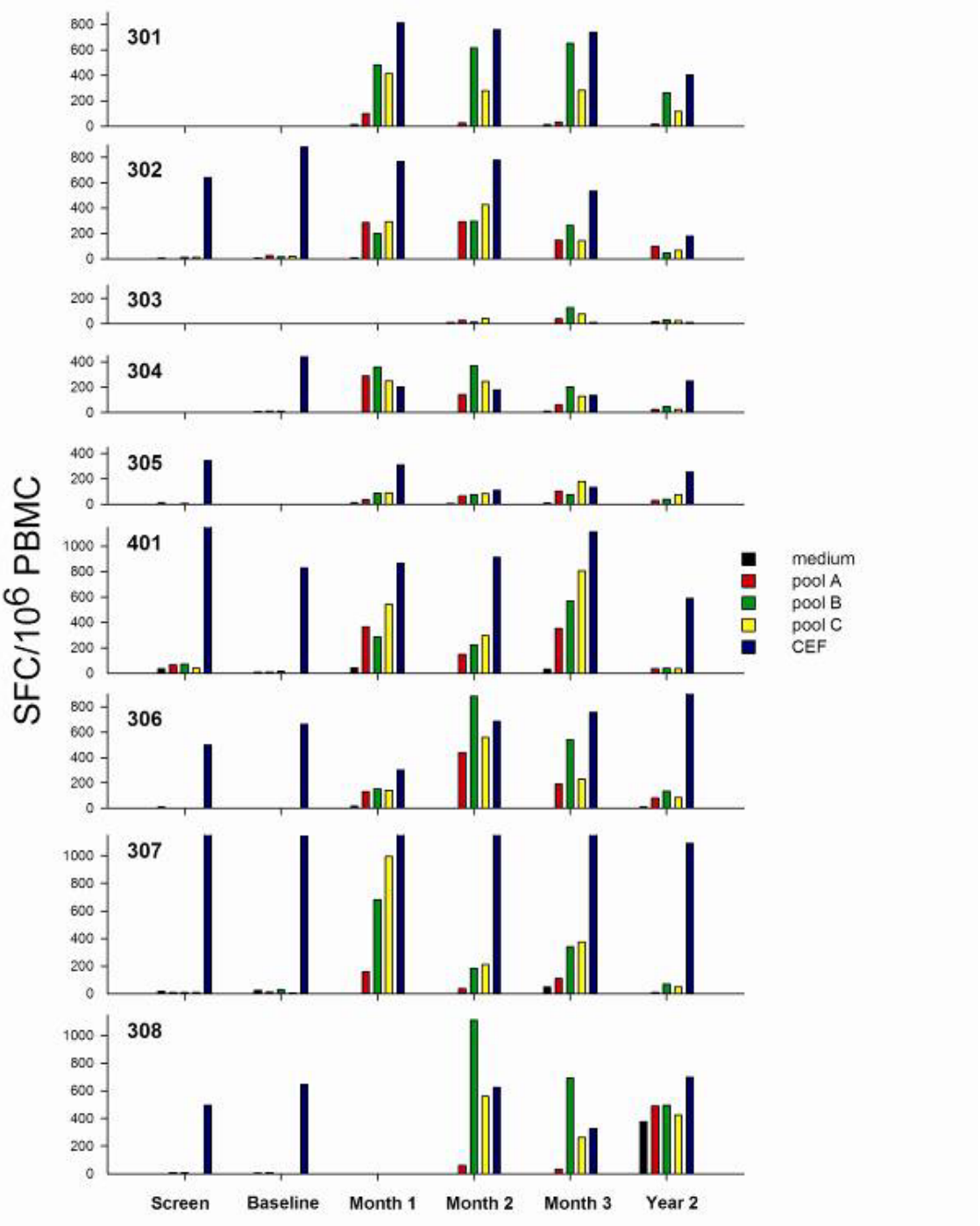
**Supplementary Figures and Tables**



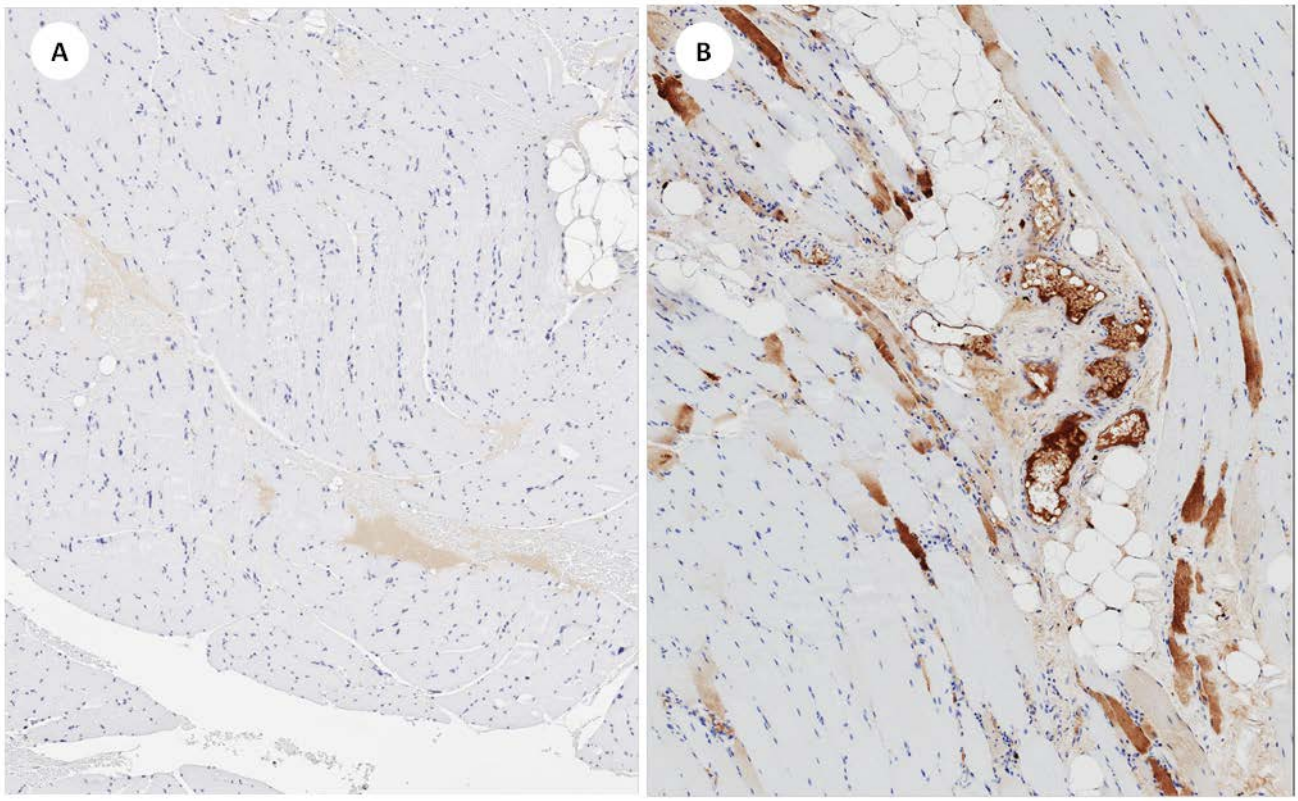

**Supplementary Figure S1** Serum AAT levels from subjects in the lower (subjects 301 and 302) and middle (subjects 304, 305 and 401) dose cohorts.



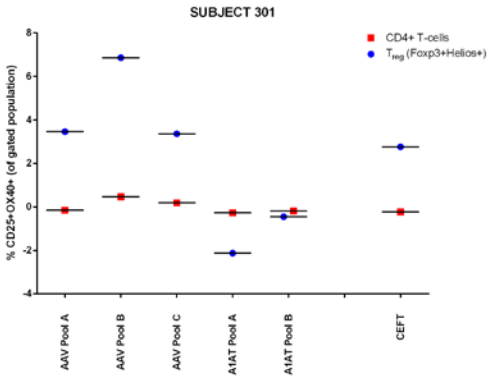

**Supplementary Figure S2** Changes in serum antibodies to herpes simplex virus over time for the nine subjects in the study (301-401). Antibody concentration was determined by ELISA using a reference standard (pooled human serum) that was assigned a titer of 8,000 U/mL. The screening values ranged from 107 to 23,291 U/mL, and no subject had a change of more than 3-fold during the first 3 months after vector administration.

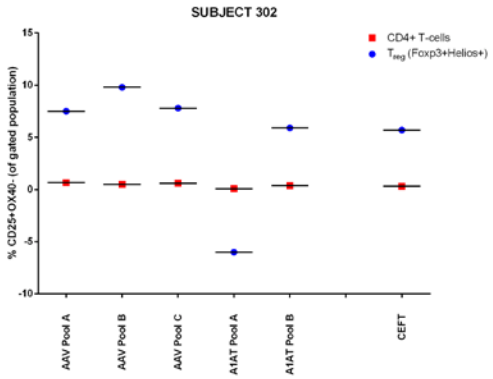


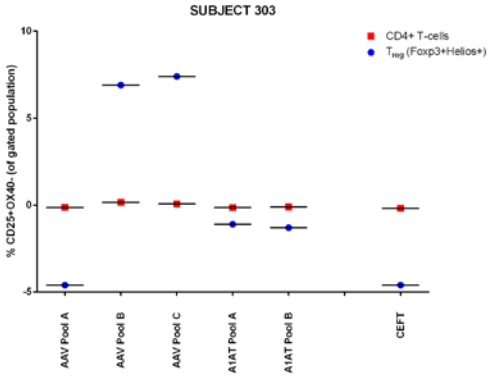

**Supplementary Figure S3** Time course of IFN-γ ELISPOT responses to pools of AAV1 capsid peptides or controls. PBMCs were obtained at screening, baseline, and 1, 2, 3, and 20 to 24 months (Year 2) after vector administration and were stimulated with each of three pools (A, B, and C) of AAV1 capsid peptides (15-mers overlapping by 10 amino acids) or with a positive control peptide pool (CEF). In general the data represents results for PBMCs from all nine patients at different times post AAV1-AAT injections, in some cases the data points are missing due to lack of PBMCs at that time point. Subject 303 did not have positive responses to CEF. Subject 308 had high background at Year 2.

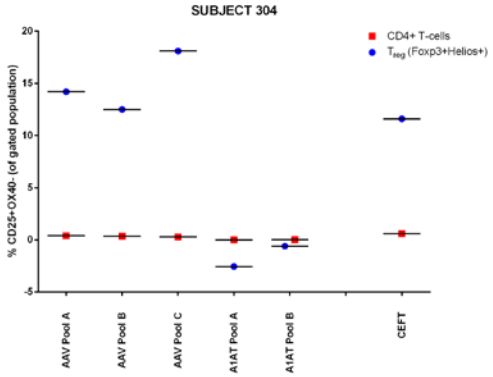


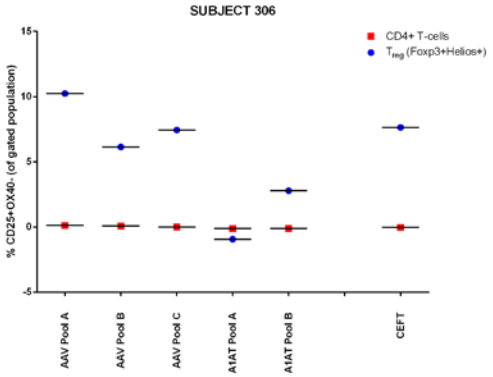

**Supplementary Figure S4** Immunohistochemistry staining for hAAT in a muscle biopsy from a normal, uninjected muscle (a) and subject injected with rAAV1-CB-hAAT (b). Images are taken at 5X magnification

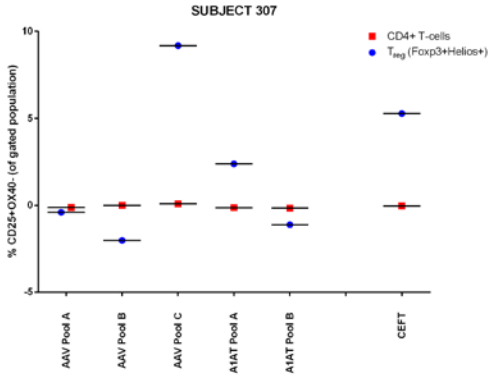

SUBJECT 301
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40+ (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT

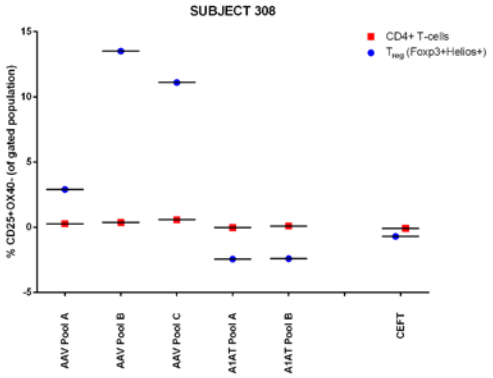

SUBJECT 302
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT

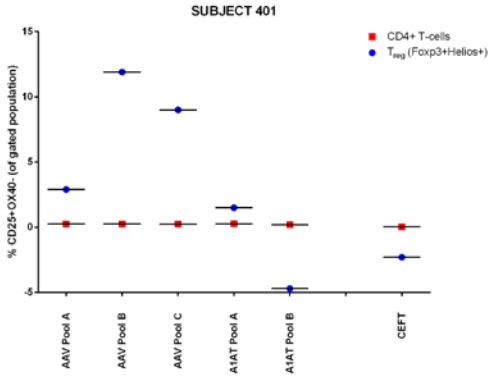

SUBJECT 303
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT


SUBJECT 304
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT


SUBJECT 306
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT


SUBJECT 307
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT


SUBJECT 308
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT


SUBJECT 401
CD4+ T-cells
Treg (Foxp3+Helios+)
% CD25+OX40- (of gated population)
AAV Pool A
AAV Pool B
AAV Pool C
A1AT Pool A
A1AT Pool B
CEFT

**Supplementary Figure S5 Antigen Specific Activation of Regulatory T-cells.** Peripheral blood mononuclear cells (PBMCs) were stimulated with AAV, AAT peptide pools or 1 ug/ml CEFT peptide. Cells were harvested at 48 hours post activation and gated for live $CD4^+$, $FOXP3^+$, and $Helios^+$ cells and then sub-gated for activation markers $OX40^+$ and $CD25^+$. Lymphocytes were gated on forward and side scatter. Live $CD4^+$ T cells were sub-gated for analysis of specific subsets as follows. Regulatory T cells were gated by co-expression 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.

Supplementary Table S1 Neutralizing antibodies to AAV1

| Visit | Dosage $6 \times 10^{11}$ vg/kg | | | Dosage $1.9 \times 10^{12}$ vg/kg | | | Dosage $6 \times 10^{12}$ vg/kg | | |
|---|---|---|---|---|---|---|---|---|---|
| | **301** | **302** | **303** | **304** | **305** | **401** | **306** | **307** | **308** |
| Screen | <5 | <5 | 80 | <5 | <5 | <5 | <5 | <5 | 160 |
| Day 14 | 5,120 | 5,120 | 40,960 | 10,240 | 5,120 | 51,20 | 20,480 | 5,120 | 20,480 |
| Month 1 | 5,120 | 2,560 | 20,480 | 10,240 | 10,240 | 10,240 | 10,240 | 2,560 | 20,480 |
| Month 3 | 20,480 | 40,960 | 20,480 | 40,960 | 20,480 | 40,960 | 81,920 | 40,960 | 81,920 |
| Month 6 | 5,120 | 20,480 | 5,120 | 20,480 | 10,240 | 10,240 | 40,960 | 40,960 | 40,960 |
| Month 12 | 10,240 | 20,480 | 5,120 | 10,240 | 10,240 | 20,480 | 20,480 | 20,480 | 40,960 |

rAAV-lacZ vectors mixed with serial dilutions of serum were used to infect Huh7 cells. Results are expressed as the reciprocal of the highest serum dilution that inhibited b-galactosidase expression by 50%.

Supplementary Table S2 Vector DNA in muscle biopsies

| Visit | Dosage $6 \times 10^{11}$ vg/kg | | | Dosage $1.9 \times 10^{12}$ vg/kg | | | Dosage $6 \times 10^{12}$ vg/kg | | |
|---|---|---|---|---|---|---|---|---|---|
| | **301** | **302** | **303** | **304** | **305** | **401** | **306** | **307** | **308** |
| **Month 3** | 293,378 | 153,255 | 282,715 | 282,715 | NS | 70,440 | 337,203 | 1,356,422 | 450,248 |
| | 543,594 | 281,856 | NS | 288,374 | NS | 80,585 | 484,615 | 2,040,711 | 249,711 |
| **Month 12** | 8,559 | 69,076 | 103,163 | 82,354 | NS | 75,982 | 46,169 | 19,527 | 255,565 |
| | 15,327 | 36,786 | 140,455 | 47,636 | NS | 36,644 | 212,273 | 336,332 | 50,945 |

Values represent vector copies per µg DNA in duplicate samples. NS = no sample.

Supplementary Table S3 Vector DNA in blood

| Visit | Dosage $6 \times 10^{11}$ vg/kg | | | Dosage $1.9 \times 10^{12}$ vg/kg | | | Dosage $6 \times 10^{12}$ vg/kg | | |
|---|---|---|---|---|---|---|---|---|---|
| | **301** | **302** | **303** | **304** | **305** | **401** | **306** | **307** | **308** |
| Screen | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Day 1 | 196,182 | 952,012 | 22,425 | 5,321,835 | 1,306,261 | 6,078,646 | 7,049,065 | 18,577,613 | 1,069,175 |
| Day 3 | 94,644 | 191,574 | 3,325 | 1,506,985 | 488,736 | 2,569,664 | 3,054,818 | 6,622,414 | 140,069 |
| Day 14 | 2,449 | 5,956 | 1,908 | 18,261 | 12,436 | 7,361 | 21,073 | 50,385 | 855 |
| Month 3 | 79 | 344 | <LOD | 1,436 | 1,565 | 412 | 6,816 | 13,227 | <LOD |
| Month 12 | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |

Values represent vector copies per µg DNA. Values below the lower limit of detection in the assay (40 vector copies per µg DNA) are indicated as <LOD.

Supplementary Table S4 Vector DNA in semen

| | **Subject Number** |
|---|---|
| **Visit** | **307** |
| Screen | <LOD |
| Day 3 | 213 |
| Day 14 | 282 |
| Month 3 | <LOD |
| Month 12 | <LOD |

Values represent vector copies per µg DNA. Values below the lower limit of detection in the assay (40 vector copies per µg DNA) are indicated as <LOD.

Supplementary Table 5 Qualitative assessment of inflammation in muscle biopsies

| Subject number | 301 | | 302 | | 303 | | 304 | | 306 | | 307 | | 308 | | 401 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 3 month biopsy | NE | 3 | 3 | 3 | 2 | 0 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 2 | 2.5 |
| 3 month mean | 3 | | 3 | | 1 | | 3 | | 3 | | 2 | | 2 | | 2.25 | |
| 12 month biopsy | 2 | 2 | 2 | 1 | 2 | 2 | 1 | 1 | 1 | 0.5 | 1 | 1.5 | 1 | 1 | 1.5 | 2 |
| 12 month mean | 2 | | 1.5 | | 2 | | 1 | | 0.75 | | 1.25 | | 1 | | 1.75 | |
| ratio 12mon/3mon | 0.67 | | 0.50 | | 2.00 | | 0.33 | | 0.25 | | 0.63 | | 0.50 | | 0.78 | |

Eight patients consented to the muscle biopsy procedure at 3 and 12 months after IM injection of rAAV1-CB-hAAT, with two tissue samples of ~0.5x1x1cm$^3$ obtained at each time point. Samples were fixed in 10% neutral buffered formalin for up to 48 hours and then transferred to 70% ethanol or PBS. After embedding in paraffin, 4 µm sections were cut for staining with hematoxylin and eosin. Stained slides were reviewed for inflammation, which was graded as severe (grade 3), moderate (grade 2), mild (grade 1) or none (0). One sample (301 sample 1) was not examined (NE) and one sample (303 sample 2) was judged to be normal muscle (grade 0). Samples that had some areas with more intense inflammation and others with less intense inflammation were assigned an intermediate grade of 0.5, 1.5 or 2.5. The mean inflammation grade for each subject at each time point was the average grade for the two samples. Seven of 8 subjects had a lower inflammation grade in the 12 month muscle biopsy than in the 3 month muscle biopsy. The group mean and standard deviation of the ratio of the inflammation grade at 12 months compared to 3 months was 0.71 ± 0.55.

Supplementary Table 6 Quantitative assessment of $CD3^+$ T cell infiltrates in muscle biopsies

| Subject number | 301 | 302 | 303 | 304 | 306 | 307 | 308 | 401 |
|---|---|---|---|---|---|---|---|---|
| Month 3 | 29.1% | 31.4% | 12.2% | 2.2% | 2.7% | 3.6% | 4.7% | 3.9% |
| Month 12 | 0.6% | 1.1% | 3.7% | 0.4% | 1.0% | 0.5% | 0.9% | 1.4% |
| Ratio 12mon/3mon | 0.02 | 0.04 | 0.30 | 0.18 | 0.37 | 0.14 | 0.19 | 0.36 |

One slide from a muscle biopsy from each of 8 subjects obtained at 3 and 12 months after IM injection of rAAV1-CB-hAAT was stained with a monoclonal antibody to CD3 followed by detection using immunoperoxidase-labeled rabbit anti-mouse IgG and analyzed using the Aperio positive pixel count image analysis program and Spectrum software. Analyses were conducted on the entire tissue section unless staining artifacts were noted, as for a small amount of precipitated chromogen, in which case these areas were excluded from analysis using the pen tool to outline the region. The standard positive pixel count algorithm default settings were used for brown chromogen quantification in three intensity ranges (220-175, 175-100, and 100-0). Pixels which were stained but did not fall into the positive-color specification were considered negative pixels. Data are reported in the table as a percentage = [100 × (number of medium and strong positive pixels) ÷ (total number of positive and negative pixels)]. For biopsies from the 8 subjects, the ratio of the percent of positive pixels at 12 months compared to the percent of positive pixels 3 months ranged from 0.02 to 0.36 with a mean ± of 0.20 ± 0.14.

Supplementary Table S7 IFN-γ ELISPOT responses to pools of AAV1 capsid peptides or controls at Month 3 and Year 2

| | | 301 | 302 | 303 | 304 | 305 | 401 | 306 | 307 | 308 |
|---|---|---|---|---|---|---|---|---|---|---|
| Media | Month 3 | 15 | 2.5 | 5 | 12.5 | 10 | 32.5 | 2.5 | 50 | 3 |
| | Year 2 | 2.5 | 0 | 5 | 0 | 2.5 | 0 | 10 | 2.5 | 377.5 |
| | Ratio | **0.17** | **0.00** | **1.00** | **0.00** | **0.25** | **0.00** | **4.00** | **0.05** | **125.83** |
| pool A | Month 3 | 32.5 | 147.5 | 37.5 | 60 | 105 | 352.5 | 190 | 110 | 33 |
| | Year 2 | 17.5 | 100 | 15 | 25 | 30 | 37.5 | 80 | 7.5 | 490 |
| | Ratio | **0.54** | **0.68** | **0.40** | **0.42** | **0.29** | **0.11** | **0.42** | **0.07** | **14.85** |
| pool B | Month 3 | 652.5 | 265 | 125 | 202.5 | 77.5 | 570 | 540 | 340 | 693 |
| | Year 2 | 265 | 47.5 | 27.5 | 47.5 | 40 | 40 | 135 | 70 | 497.5 |
| | Ratio | **0.41** | **0.18** | **0.22** | **0.23** | **0.52** | **0.07** | **0.25** | **0.21** | **0.72** |
| pool C | Month 3 | 282.5 | 142.5 | 75 | 127.5 | 180 | 807.5 | 227.5 | 375 | 263 |
| | Year 2 | 117.5 | 67.5 | 22.5 | 25 | 77.5 | 35 | 85 | 50 | 425 |
| | Ratio | **0.42** | **0.47** | **0.30** | **0.20** | **0.43** | **0.04** | **0.37** | **0.13** | **1.62** |
| CEF | Month 3 | 740 | 535 | 10 | 135 | 132.5 | 1115 | 755 | 1225 | 328 |
| | Year 2 | 405 | 180 | 10 | 250 | 255 | 590 | 965 | 1090 | 700 |
| | Ratio | **0.55** | **0.34** | **1.00** | **1.85** | **1.92** | **0.53** | **1.28** | **0.89** | **2.13** |

Values at Month 3 and Year 2 (Month 20-24) are spot-forming cells per $10^6$ PBMC, and the ratio values are Year 2 values ÷ Month 3 values. Subject 308 had high background at Year 2. For the other 8 subjects, responses to AAV pools A, B and C (total of 24 values) were all lower at Year 2 than Month 3, and the ratio of Year 2 vs. Month 3 responses (mean ± SD) was 0.36 ± 0.21 for pool A, 0.26 ± 0.14 for pool B and 0.30 ± 0.16 for pool C. Statistical evaluation using analysis of variance (ANOVA) for these 24 values determined an F-value of 21.353 ($p<0.0001$), with a significant difference between Month 3 and Year 2 (mean difference 190, critical difference 82.987, $p<0.0001$). There were no significant differences between Year 2 and Month 3 responses to CEF or the medium control, with a Year 2 vs. Month 3 ratio of 1.04 ± 0.60 for CEF and 0.68 ± 1.38 for the medium control.

Supplementary Table S8 Immunohistochemistry staining conditions for muscle sections.

| Antigen Retrieval | 1st antibody | Vendor, Cat# | Incubation time | 2nd antibody |
|---|---|---|---|---|
| Trypsin, 5min | AAT | Fitzgerald, 20R-AR009 | 60min | Mach2 Rb HRP polymer |
| Trilogy, 25min | CD3 | DAKO, A0452 | 60min | Mach2 Rb HRP polymer |
| Trilogy, 25min | CD4 | DAKO, m7310 | 60min | Mach2 Ms HRP polymer |
| Trilogy, 25min | CD8 | DAKO, m7103 | 60min | Mach2 Ms HRP polymer |
| Trilogy, 25min | CD20 | DAKO, m0755 | 60min | Mach2 Ms HRP polymer |
| Trilogy, 25min | CD68 | DAKO, M0876 | 60min | Mach2 Ms HRP polymer |
| Citra, 30min | CD274 | Novus Biologicals, NBP1-03220 | O/N | Mach2 Rb HRP polymer |
| Citra, 30min | CD279 | Novus Biologicals, NBP1-88104 | O/N | Mach2 Rb HRP polymer |

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