Supplementary Data

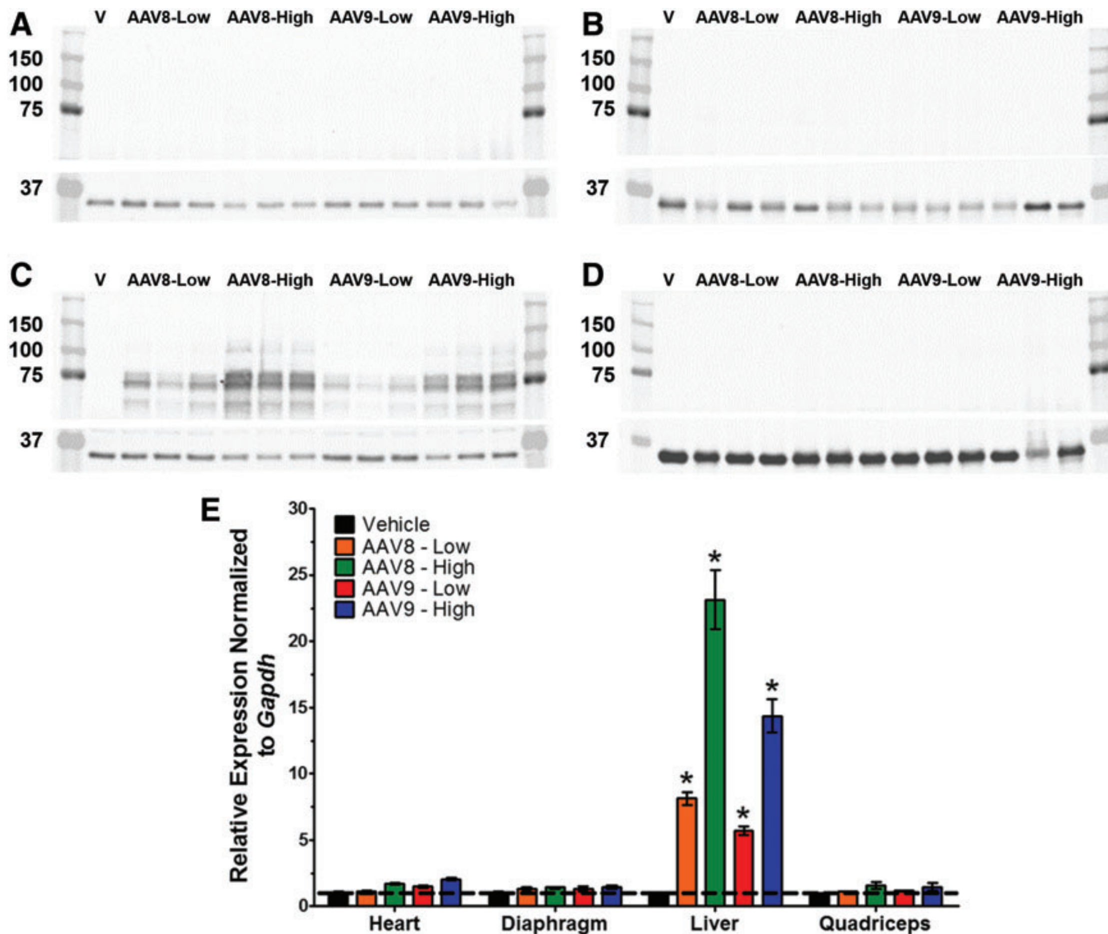
SUPPLEMENTARY MATERIALS AND METHODS

Western blot analysis

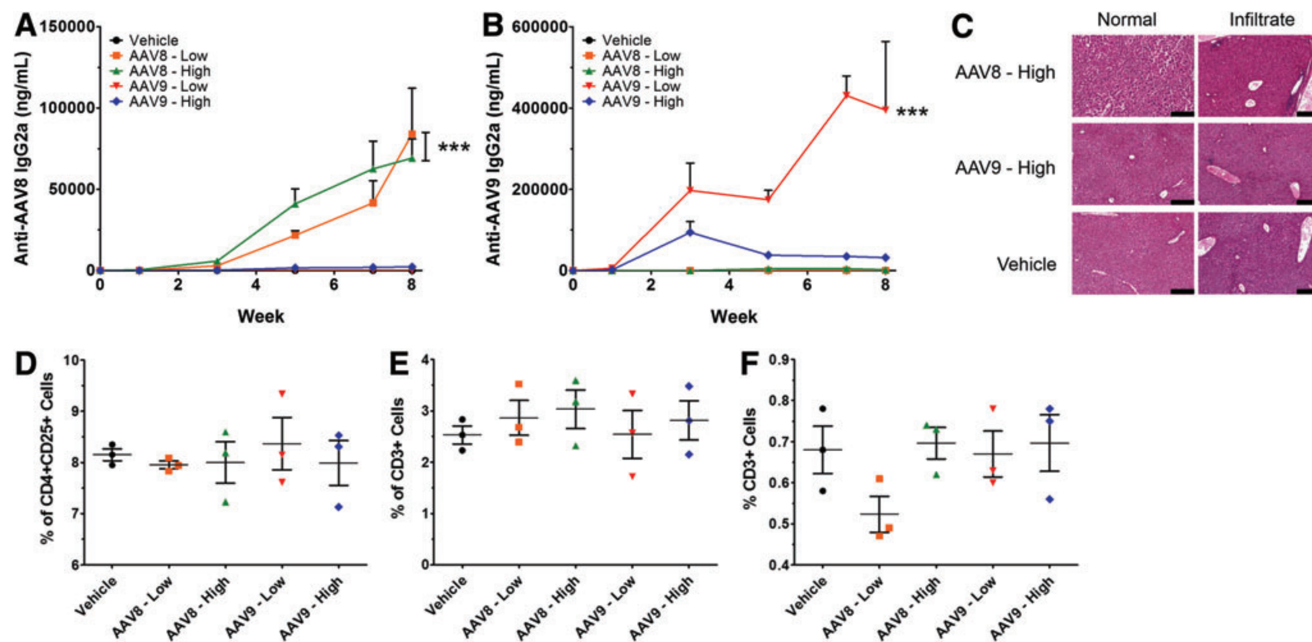
Protein concentration was quantified using detergent-compatible (DC) protein assay reagents following the manufacturer's instructions (Bio-Rad: 5000111). Samples were denatured at 95°C for 10 min, and 30 μg of total protein was electrophoresed on a 10% Tris-HCl gel. Polyvinylidene difluoride (PVDF) transfers were probed with a 1:100,000 dilution of a mouse monoclonal anti-human acid alpha-glucosidase (GAA) antibody[1] or 1:10,000 dilution of a rabbit monoclonal anti-mouse GAPDH antibody (Cell Signaling Technology: 14C10). Protein was visualized using the LI-COR imaging system with goat antimouse 800CW (LI-COR: 926-32210) and goat antirabbit 800CW (LI-COR: 926-32211) secondary antibodies.

Quantitative reverse transcription polymerase chain reaction

Heart, diaphragm, liver, and quadriceps were homogenized in TRIzol Reagent (Thermo: 15596-018). After phenol-chloroform extraction, RNA was



Supplementary Figure S1. Transgene expression specificity in liver-directed gene therapy-treated *Gaa*$^{-/-}$ mice. Western blot analysis for GAA in the **(A)** heart, **(B)** diaphragm, **(C)** liver, and **(D)** quadriceps in 0.5 (low) or 5×10^{12} (high) vg/kg AAV8- or AAV9-LSP-coGAA-injected *Gaa*$^{-/-}$ mice. Lane V represents a vehicle-injected *Gaa*$^{-/-}$ mouse (negative control) with three replicates of each dose represented. GAA is processed as 110, 95, and 76 kDa precursors with the 70 kDa isoform representing the mature, active enzyme. An amount of 30 μg of lysate was electrophoresed on a 10% Tris-HCl gel with GAPDH (37 kDa) serving as the loading control. **(E)** RT-qPCR analysis of transgene expression in the heart, diaphragm, liver, and quadriceps normalized to *Gapdh*. Kruskal–Wallis test results with significance levels of pairwise comparisons to vehicle are shown. Results represented as mean ± SEM; $n = 3$/group; $^{*}p < 0.05$ versus vehicle. AAV, adeno-associated virus; GAA, acid alpha-glucosidase; LSP, liver-specific promoter.

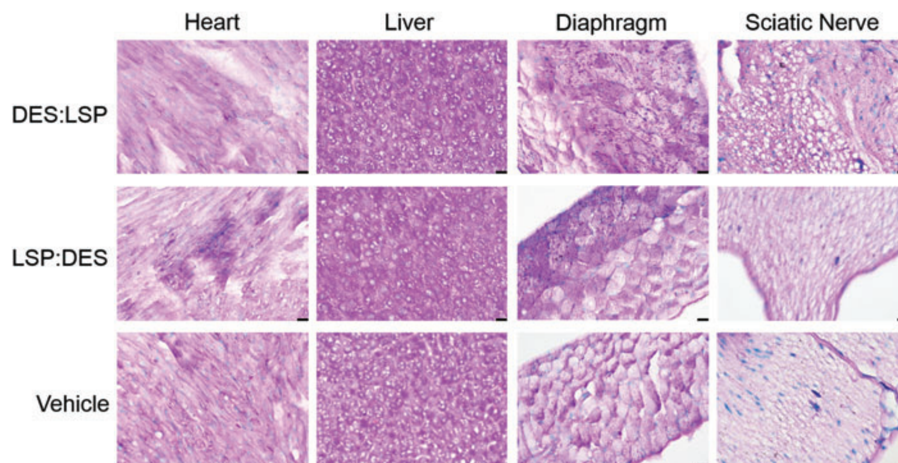


Supplementary Figure S2. Anti-capsid response, liver histology, and splenocyte activation in liver-directed gene therapy-treated *Gaa*$^{-/-}$ mice. **(A)** Anti-AAV8 and **(B)** anti-AAV9 IgG2a titers observed during gene transfer after 0.5 (low) or 5×10^{12} (high) vg/kg of AAV8- or AAV9-LSP-coGAA compared with vehicle. Two-way ANOVA results with significance levels of Bonferroni multiple comparison posttest compared with vehicle are shown. **(C)** Hematoxylin and eosin staining of the liver after 8 weeks in 5×10^{12} vg/kg AAV8- or AAV9-LSP-coGAA-, or vehicle-treated mice. Images are representative of 4–6 mice per group. Scale bars represent 200 μm. **(D–F)** FACS analysis of splenocytes. **(D)** % of CD3+CD4+CD25+FoxP3+ T-cells, **(E)** % of CD3+CD4+CD69+ T-cells, and **(F)** % of CD3+CD8+CD69+ T-cells. Results represented as mean ± SEM; $n=3$–6/group; ***$p<0.001$ versus vehicle. AAV, adeno-associated virus.

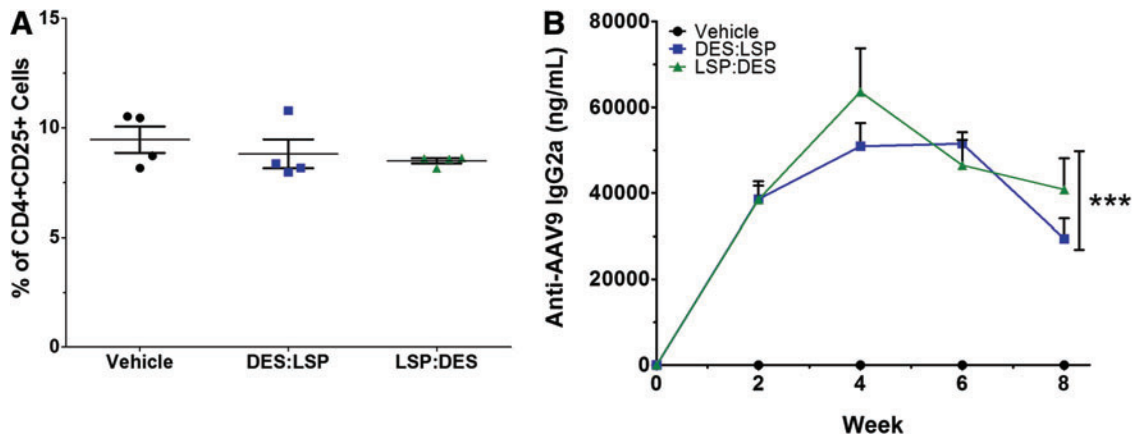
isolated using the Qiagen RNeasy kit (Qiagen: 74104). RNA concentration was determined using NanoDrop spectrophotometry (Thermo). Complementary DNA (cDNA) synthesis and qPCR were performed as described previously.[2] Primers designed for codon-optimized GAA (coGAA) were 5′-TCTCCCGTGCTGGAAGAAAC and 3′-AGTCGAA TCTGCTGTTGGGG. Gene expression was normalized to *Gapdh* using previously described primers.[2] Change in gene expression relative to *Gapdh* was determined using the ΔΔCt method.

Fluorescence-activated cell sorting

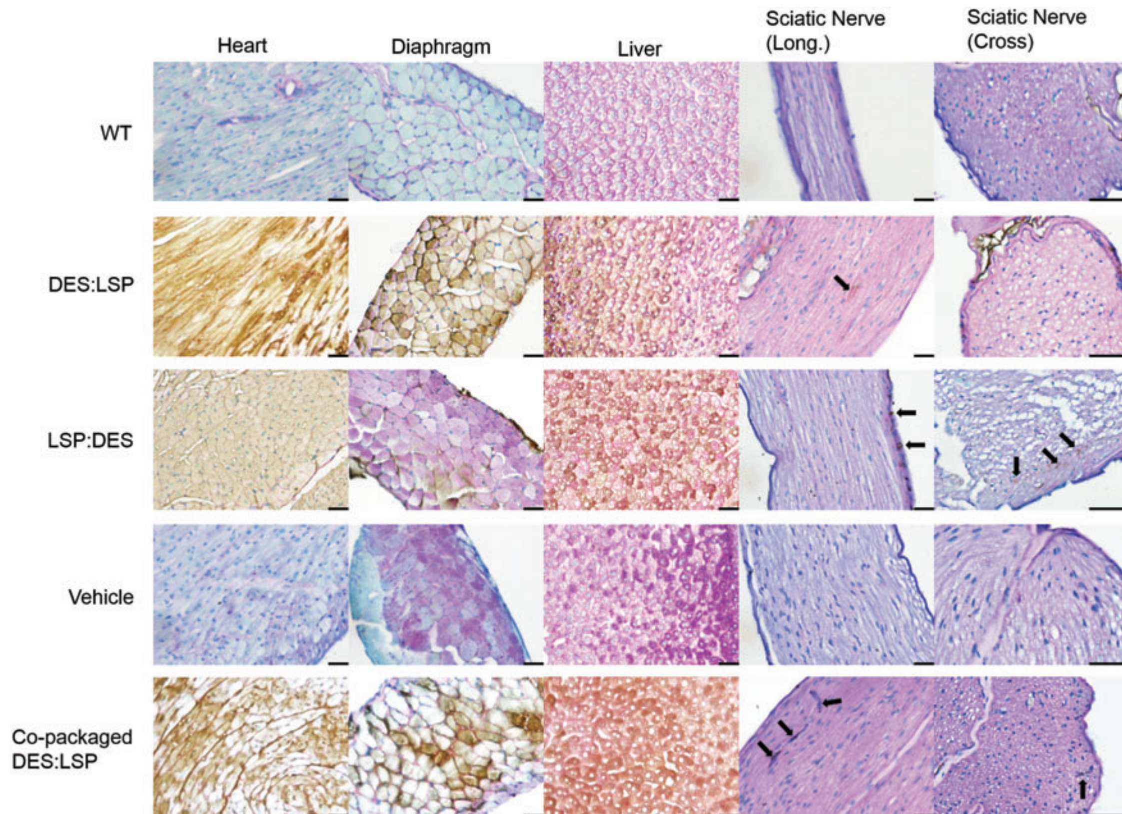
Spleens were strained through a 40 μm nylon mesh strainer to obtain a single-cell suspension. Before labeling, cells were blocked with Fc block



Supplementary Figure S3. Histologic pathology persists after subtherapeutic dose of combined AAV9 vectors in *Gaa*$^{-/-}$ mice. Periodic acid-Schiff of glycogen 8 weeks post-gene transfer of 0.5×10^{13} vg/kg of admixed DES:LSP, LSP:DES, or vehicle-treated mice. Images are representative of 4 mice. Scale bars represent 20 μm.



Supplementary Figure S4. FACS analysis of splenocytes and anticapsid response after subtherapeutic dose of combined AAV9 vectors in $Gaa^{-/-}$ mice. **(A)** % of CD3+CD4+CD25+FoxP3+ T-cells 8 weeks post-gene transfer of 0.5×10^{13} vg/kg of admixed DES:LSP, LSP:DES, or vehicle-treated mice. **(B)** Anti-AAV9 IgG2a titers observed during gene transfer. Two-way ANOVA results with significance levels of Bonferroni multiple comparison posttest compared with vehicle are shown. Results represented as mean ± SEM; $n=4$/group; $***p<0.001$ versus vehicle. DES, desmin promoter; GAA, acid alpha-glucosidase; LSP, liver-specific promoter; DES:LSP (9 DES: 1 LSP of total dose); LSP:DES (9 LSP: 1 DES of total dose).



Supplementary Figure S5. GAA immunostaining and PAS staining in dual-vector-treated $Gaa^{-/-}$ mice. GAA immunostaining (brown; black arrows) and PAS of glycogen (purple) in heart, diaphragm, and liver with longitudinal and cross sections of sciatic nerve 8 weeks post-gene transfer of 5×10^{13} vg/kg of admixed DES:LSP, LSP:DES, and copackaged DES:LSP in comparison to age-matched wild-type and vehicle-treated $Gaa^{-/-}$ mice. Scale bars represent 20 μm. Images are representative of 4–6 mice per group. DES, desmin promoter; GAA, acid alpha-glucosidase; LSP, liver-specific promoter; DES:LSP (9 DES: 1 LSP of total dose); LSP:DES (9 LSP: 1 DES of total dose); PAS, Periodic acid-Schiff.

(2.4G2; BD Biosciences). Antibodies to Alexa Fluor 700-CD4 (GK1.5), eFluor 450-CD8 (53-6.7), Pe-Cy7-CD25 (PC61.5), FITC-CD69 (H1.2F3), and APC-FoxP3 (FJK-16s) were from eBioscience. Antibody to PE-CD3 (17A2) was from BioLegend. Intracellular staining was performed following manufacturer's protocol (eBioscience: 72-5775-40). FACS was performed on an LSRII (BD Biosciences) and analyzed using FCS Express 4 (De Novo Software).

Histology

Heart, diaphragm, liver, and sciatic nerve were fixed as indicated in the main text. Hematoxylin and eosin staining was performed using reagents from SurgiPath (Leica Biosystems) using standard procedures. For GAA IHC with Periodic acid-Schiff (PAS), anti-GAA immunostaining was performed first using a polyclonal rabbit anti-human GAA (Covance) at 1:500 and developed using biotinylated antirabbit IgG secondary antibody at 1:200 (Vector Laboratories) coupled with a Vectastain Elite ABC Kit (Vector Laboratories: PK-6100). PAS was then performed on the GAA-labeled sections according to manufacturer's protocol indicated in the main text.

SUPPLEMENTARY REFERENCES

1. Maga JA, Zhou J, Kambampati R, et al. Glycosylation-independent lysosomal targeting of acid α-glucosidase enhances muscle glycogen clearance in Pompe mice. J Biol Chem 2013;288:1428–1438.
2. Todd AG, McElroy JA, Grange RW, et al. Correcting neuromuscular deficits with gene therapy in Pompe disease. Ann Neurol 2015;78:222–234.