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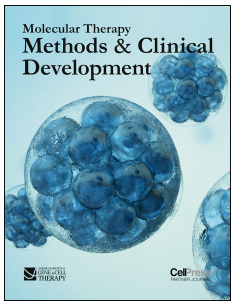

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Low-dose liver targeted gene therapy for Pompe disease enhances therapeutic efficacy of ERT via immune tolerance induction

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ABSTRACT

Pompe disease results from acid α-glucosidase (GAA) deficiency and enzyme replacement therapy (ERT) with recombinant human (rh) GAA has clinical benefits, although its limitations include the short half-life of GAA and the formation of antibody responses. The present study compared the efficacy of ERT against gene transfer with an adeno-associated viral (AAV) vector containing a liver-specific promoter. GAA knockout (KO) mice were administered either a weekly injection of rhGAA (20 mg/kg), or a single injection of AAV2/8-LSPhGAA ($8x10^{11}$ vector genomes (vg)/kg). Both treatments significantly reduced glycogen content of the heart and diaphragm. While ERT triggered anti-GAA antibody formation, there was no detectable antibody response following AAV vector administration. The efficacy of 3 lower dosages of AAV2/8-LSPhGAA was evaluated in GAA-KO mice, either alone or in combination with ERT. The minimum effective dose (MED) identified was $8x10^{10}$ vg/kg to reduce glycogen content in the heart and diaphragm of GAA-KO mice. A 3-fold higher dose was required to suppress antibody responses to ERT. Efficacy from liver gene therapy was slightly greater in male mice than in females. Vector dose correlated inversely with anti-GAA antibody formation, while higher vector doses suppressed previously formed anti-GAA antibodies as late as 25 weeks after the start of ERT and achieved biochemical correction of glycogen accumulation. In conclusion, we identified the MED for effective AAV2/8-LSPhGAA-mediated tolerogenic gene therapy in Pompe disease mice.

INTRODUCTION

Pompe disease is an inherited rare disorder caused by mutations in the gene for the enzyme acid alpha glucosidase (GAA, >1 in 40,000 births) that affects the heart and skeletal muscles, and is often fatal.[1] Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) has been shown to decrease heart size, maintain normal heart function, improve muscle function, tone, and strength, and reduce glycogen accumulation.

Although ERT has prolonged survival in the majority of patients with infantile Pompe disease, many patients have died or remained very weak despite compliance with ERT. Among the poor responders to ERT were many cross-reacting immune material negative (CRIM-negative) patients, who lack any residual GAA protein and who formed high, sustained anti-rhGAA IgG antibody titers (HSAT). Patients with HSAT demonstrated greatly increased mortality, in comparison with patients who formed no or low titer antibodies.[2] Furthermore, suppressing anti-rhGAA antibody formation with immunosuppression significantly prolonged the survival of CRIM-negative infants, although immunosuppression has associated risks.[3,4] A small minority of adult patients with late-onset Pompe disease (LOPD) also formed HSAT during ERT, which in some cases were associated with reduced efficacy. [5,6]

Multiple preclinical experiments have demonstrated the ability of gene therapy to prevent antibody formation in mice with Pompe disease.[7-10] Preventing HSAT also reduced mortality from hypersensitivity that had occurred during ERT in GAA-knock out (KO) mice, while ERT was efficacious only in the setting of immune tolerance to GAA following AAV vector administration.[8] We and others demonstrated that AAV vector mediated gene transfer consistently induced immune tolerance to GAA by expressing GAA exclusively in the liver and by activating regulatory T cells in preclinical experiments.[7-11]

The efficacy from ERT in Pompe disease is limited by the short half-life of GAA and the formation of antibody responses that interfere with the uptake of GAA. We hypothesized that

liver-specific expression of GAA with a recombinant (r) AAV8 vector expressing human GAA under the transcriptional control of a liver specific promoter (AAV2/8-LSPhGAA[12]) would suppress the antibody response, continually secrete GAA in the blood, and improve efficacy in comparison with ERT. Previous studies suggested that the efficacy of this rAAV8 vector at a low dose[12] ($2x10^{10}$ vector genomes, vg, equivalent to $8x10^{11}$ vg/kg body weight) was comparable to long-term ERT[13,14] with regard to biochemical correction. Importantly, a higher vector dose ($4x10^{12}$ vg/kg) reduced glycogen in skeletal muscle by 70% more than intensive ERT in GAA knockout (KO) mice.[7,14] The current study directly compares ERT with AAV2/8-LSPhGAA, and supported a successful investigational new drug (IND) application to the Food and Drug Administration in anticipation of a clinical trial of liver depot gene therapy in Pompe disease.

RESULTS

AAV8-GAA liver gene transfer is as effective as ERT and prevents anti-GAA antibody formation

We hypothesized that liver depot gene therapy for Pompe disease could potentially improve clinical outcomes (Fig. 1). Therefore, we directly compared the efficacy of intensive ERT with AAV2/8-LSPhGAA gene transfer at the established[12] low dose. GAA-KO mice were assigned (both male and female; Fig. 1A and Table 1) to receive either a weekly injection of rhGAA for intensive ERT[15] (20 mg/kg/week; n=10), or a single injection of AAV2/8-LSPhGAA for low dose gene therapy ($8x10^{11}$ vg/kg; n=10). The primary endpoints included GAA activity and glycogen content in the tissues and anti-GAA antibody formation. In both ERT- and gene therapy-treated animals, GAA activity was significantly increased in liver following both treatments, whereas GAA activity was higher without reaching statistical significance in the heart and muscles following gene therapy (Fig. 1B). Glycogen content was reduced by both treatments in the heart and diaphragm, demonstrating that glycogen content is a more sensitive measure of

biochemical correction than GAA activity (Fig. 1C) as previously observed.[12] While ERT provoked anti-GAA antibody formation, there was no detectable antibody response following AAV vector administration (Fig. 1D). GAA activity was continuously elevated in the blood of mice following a single injection of vector, and not detectable 7 days following ERT (Fig. 1E). The left ventricle mass was reduced significantly following either ERT or vector injection, demonstrating the reversal of cardiac hypertrophy (Fig. 1F). As expected, AAV2/8-LSPhGAA demonstrated slightly greater efficacy in male mice than in females, including higher liver GAA activity and lower diaphragm glycogen (Fig. S1). Additionally, female mice had higher anti-GAA IgG1 following ERT, in comparison with males (Fig. S1). Thus, gene therapy with AAV2/8-LSPhGAA at a dose of $8x10^{11}$ vg/kg was confirmed to have similar efficacy to ERT in both sexes, with the added benefit of avoiding anti-GAA immune responses.

Definition of the minimum effective dose (MED) of liver depot gene therapy for Pompe disease

Next we evaluated the biochemical efficacy of 4 lower dosages of AAV2/8-LSPhGAA in GAA-KO mice, either alone or in combination with ERT (Fig. 2A; Table 1). No biochemical correction was observed following administration of $6x10^{9}$ vg/kg (not shown). The highest dose ($2x10^{11}$ vg/kg) significantly increased GAA activity in the heart, either with or without ERT (Fig. 2B). Administration of the highest vector dose reduced glycogen content of the heart to a greater extent in male mice, than in female mice (Fig. S2). Lower vector dosages were associated with lower GAA activity in the heart and skeletal muscles that did not achieve statistical significance, in comparison with no vector administration (Fig. 2B). However, AAV2/8-LSPhGAA significantly reduced glycogen content in the heart and diaphragm of GAA-KO mice at a dose of $8x10^{10}$ vg/kg (Fig. 2C, $p<0.01$), which demonstrated that the glycogen storage in muscle associated with Pompe disease was substantially cross-corrected by GAA secretion from liver.

Furthermore, administering ERT by itself had no significant effect on the glycogen content of quadriceps, but ERT following administration of the second highest dose ($8x10^{10}$ vg/kg) of AAV2/8-LSPhGAA reduced glycogen content of quadriceps by 38% ($p<0.05$; Fig. 2D), indicating that gene therapy with AAV2/8-LSPhGAA could possibly have a synergistic effect with ERT. Therefore, the MED with regard to biochemical correction was established as $8x10^{10}$ vg/kg.

Administration of low dose AAV2/8-LSPhGAA **reduces humoral responses to GAA following an immune challenge with ERT**

Total IgG and IgG subclasses were quantified in the serum of mice obtained one week following the last dose of ERT. In this experiment IgG2a and IgG3 were not detected (not shown). As expected[16], we observed an increased level of total IgG specific for human GAA following 4 doses of ERT without AAV, in comparison with mice that were untreated (Fig. 3A, Vector Dose "0"). Similarly, significant increases in anti-GAA IgG1 were observed following ERT alone, or administration of a vector dose of $8x10^{10}$ vg/kg (Fig. 3B). Interestingly, at a dose of $2x10^{11}$ vg/kg vector administration prevented significantly increased total IgG or IgG1 in response to ERT (Fig. 3A-B). Surprisingly, the administration of vector alone at the dose of $2x10^{10}$ vg/kg induced a humoral immune response in 4 out of 8 mice (Fig. 3A-B). However, the administration of vector alone at the dose of $8x10^{10}$ vg/kg induced a humoral response in only 2 of 9 mice, and the dose of $2x10^{11}$ vg/kg did not induce a humoral response. When ERT was administered following a vector dose of $8x10^{10}$ vg/kg, a humoral response was induced in 7 of 9 mice; however, following a vector dose of $2x10^{11}$ vg/kg a humoral response was induced in only 2 of 9 mice. Furthermore, in no group was total IgG or IgG1 increased to a greater extent following vector administration, in comparison with ERT alone (Fig. 3A-B). Taken together these data indicated that the highest dose of vector delivered to the liver prevented the induction of

humoral immune responses to GAA. Thus, the MED with regard to immune tolerance induction was established at $2x10^{11}$ vg/kg.

Anti-GAA IgG1 was the isotype most prevalent in mice that received ERT combined with a low vector dose. Interestingly, we also observed increased levels of IgG2b and IgG2c following the administration of vector alone at the $2x10^{10}$ vg/kg dose, in comparison with mice that received vector and ERT (Fig. 3C-D). The vector dose of $2x10^{10}$ vg/kg induced a humoral response characterized by high titers of IgG1, IgG2b and IgG2c (greater in male than in female mice; Fig. S3), whereas a higher vector dose generally prevented IgG formation. This unique isotype profile was not observed in the other groups treated with vector or ERT alone or in combination. This unique profile was most pronounced in male mice, in comparison with female mice (Supp. Fig 3). Notably, an isotype switch from IgG1 to IgG2b and IgG2c isotypes has been associated with the induction of peripheral tolerance mediated by the liver[17], or immune deviation from a Th2 to a Th1 response.[16]

Induction of immunological tolerance to GAA following gene transfer correlates with vector genome copy number in liver

The induction of liver-mediated transgene tolerance is highly dependent upon a robust liver transduction.[10,12] Vector genome copy number (VGCN) in liver represents a surrogate for liver transduction. To correlate antibody formation in mice treated with AAV8-LSP-hGAA with the VGCN, we arbitrarily divided mice in three groups: 1) those with less than 0.01 copy, considered as the limit of detection of VGCN measurement; 2) those with between 0.01 and 1 copy, and 3) those with more than 1 copy per diploid genome. Total IgG and IgG subclasses were quantified in the three groups, and compared to untreated mice and to mice treated with ERT without liver gene transfer. As previously shown, ERT treatment in the absence of the vector induced IgG

(Fig. 3E) and IgG1 (Fig. 3F) against rhGAA, which could not be prevented by liver transduction corresponding to less than 1 copy of vector per diploid genome. Conversely, the treatment with AAV8-LSP-hGAA doses that resulted in more than 1 copy per diploid genome (>1) prevented the induction of a significant humoral immune response against the transgene. Furthermore, intermediate transduction of 0.01 to 1 copy allowed significant formation of IgG2c (Fig. 3H). The formation of IgG2c was significantly increased in the presence of intermediate vector transduction without ERT, in comparison with intermediate transduction with ERT (Fig. 3H).

Eradication of anti-GAA humoral immunity and long-term efficacy of AAV2/8-LSP-hGAA gene transfer

The long-term effect of AAV2/8-LSP-hGAA in combination with ERT was evaluated with regard to immune tolerance induction (Fig. 4; Table 1), given the experience that vector administration following the start of ERT might fail to prevent anti-GAA formation.[16] The ability of AAV2/8-LSP-hGAA to suppress anti-GAA antibody formation following exposure to ERT was evaluated by administering the vector either 5 or 25 weeks following the initiation of ERT (Fig. 4A). Initially the mice were treated with two doses of ERT according to standard recommendations (20 mg/kg every other week). At Week 5 mice were treated with an additional dose of ERT (Grp 1) or with AAV2/8-LSP-hGAA at a highly effective dose to evaluate the antibody response to each agent. Early monitoring revealed that ERT (Grp 1; Fig. 4B) provoked significantly higher anti-GAA formation, in comparison with AAV2/8-LSP-hGAA (Grp 2; Fig. 4B), whereas the administration of only 2 doses of ERT did not provoke anti-GAA formation (Grp 3; Fig. 4B). Long-term monitoring revealed that the administration of AAV2/8-LSP-hGAA at Week 5 suppressed anti-GAA formation in response to two subsequent doses of ERT at Week 17 and 19 (Grp 2; Fig. 4B). However, administration of ERT at Week 17 and 19 provoked significantly elevated anti-

GAA by 23 weeks in mice that had received only ERT (Grp 3; Fig. 4B). Thus, administration of AAV2/8-LSP-hGAA as late as Week 5 following the initiation of ERT was sufficient to induce long-term immune tolerance to rhGAA.

We further evaluated the ability of AAV2/8-LSP-hGAA to suppress previously formed antibody responses against ERT. AAV2/8-LSP-hGAA was administered at Week 25 to suppress previously formed anti-GAA, reducing the anti-GAA IgG1 to background levels by Week 36 (Grp 3; Fig. 4B). Initially, ELISA confirmed that 10 of 10 mice were positive for anti-GAA at Week 23, whereas only 1 of 9 mice were positive at Week 36 following AAV2/8-LSPhGAA administration (Grp 3; Fig. 4B). When all 3 groups were challenged with ERT at week 31, GAA-KO mice treated with ERT only formed significantly increased anti-GAA at Week 36 (Grp 1; Fig. 4B). GAA-KO mice that were treated with AAV2/8-LSP-hGAA at Week 5 developed increased Rotarod latency in comparison with all other groups (Grp 2, Fig. 4C), which demonstrated the importance of early vector treatment to improve muscle function. Both vector-treated groups had improved biochemical correction after 36 weeks, in comparison with mice treated with ERT alone (Fig. 4D-E). GAA activity was significantly increased in the heart, diaphragm, and soleus of GAA-KO mice following administration of AAV2/8-LSP-hGAA, in comparison with ERT-treated or untreated mice (Fig. 4D). Vector administration significantly reduced glycogen content in multiple skeletal muscles of vector treated mice (Fig. 4E), and glycogen content was again a more sensitive measure of biochemical correction than GAA activity (Fig. 4D) as previously demonstrated.[12] The soleus demonstrated the greatest elevation of GAA (Fig. 4D) and reduction in glycogen content (Fig. 4E) following vector administration. This observation was consistent with improved response of type I muscles to GAA replacement[14], in comparison with other muscles examined that were comprised mainly of type II myofibers. As expected[16], AAV2/8-LSPhGAA-treated mice demonstrated slightly greater efficacy in males than in females, achieving higher GAA activity and lower glycogen content (Fig. S4). In general the degree of

biochemical correction achieved by early and late vector injection was equivalent with regard to reducing glycogen content, confirming the efficacy of suppression of anti-GAA with AAV2/8-LSP-hGAA (Fig. 4E).

Circulating IgG isotypes in the three groups were quantified at Week 36, and ranked according to the degree of elevation (Fig. 5; Supplemental Table 1). As expected, ERT-only mice formed the highest total IgG with 9 out of 9 mice generating elevated total IgG (Grp 1; Fig. 5A) and IgG1 (Fig. 5B). Administering AAV2/8-LSP-hGAA at Week 5 reduced total IgG and IgG isotypes, with 6 out of 9 mice showing detectable IgG1 levels, below 0.25 µg/ml (Grp 2; Fig. 5B; Supp. Table 1). Vector- treated mice had significantly lower IgG (Grp 2 and 3; Fig. 5C) and IgG1 (Grp 2 and 3; Fig. 5D) responses, in comparison with the ERT-only Grp 1. Finally, AAV2/8-LSP-hGAA at Week 25 resulted in elevated total IgG (Grp 3; Fig. 5C) or IgG1 (Grp 3; Fig. 5D) in only three out of 9 mice according to the ranking scale, thus indicating an efficient eradication of the immune response 11 weeks following vector administration. Interestingly, following AAV2/8-LSP-hGAA at Week 25 only very low titers of IgG1 were observed, along with IgG2b and IgG2c (Grp 3; Fig. S5). IgG1 was elevated to >10 µg/ml in only one of 9 Grp 3 mice (Fig. 5B), which would correspond to an ELISA titer >1:1,600 (Fig. S6) that would be expected to interfere with efficacy.[8] Together, these data suggest that AAV vector mediated liver gene transfer for GAA can eradicate established immune responses to the enzyme and enhance efficacy of GAA replacement and, consequently, clinical outcome in Pompe disease.

DISCUSSION

This study modeled the clinical translation of a gene therapy approach aimed at preventing IgG antibody formation in patients at risk for HSAT, and addressed important questions related to the successful filing of an IND. This strategy induced immune tolerance to ERT through liver

specific expression of GAA. Furthermore, liver expression of GAA treats Pompe disease by the continuous secretion of GAA from the liver accompanied by receptor-mediated uptake in the heart and skeletal muscles. Three experiments investigated the effectiveness of liver depot gene therapy, and its interactions with ERT. A single dose of AAV2/8-LSPhGAA was as effective as intensive, weekly ERT (Fig. 1). This direct comparison confirmed previous experiments that suggested that gene therapy with a very low amount of AAV2/8-LSPhGAApA, equivalent to $8x10^{11}$ vg/kg body weight, was as effective as ERT[13,14], and more precisely quantitated the reduction in glycogen content following GAA replacement than a previous comparison between ERT and gene therapy.[18] We established the MED for biochemical correction with AAV2/8-LSPhGAA ($8x10^{10}$ vg/kg), which is approximately 10-fold lower than previous data suggested (Fig, 2).[8,9] The MED for immune tolerance is 2.5-fold higher ($2x10^{11}$ vg/kg). As previously observed glycogen content was a more sensitive indicator of biochemical correction than GAA activity, because glycogen content was significantly reduced in muscles where GAA activity was not significantly increased (Fig. 1-3).[12,19] Finally, the ability of AAV2/8-LSPhGAA to suppress or eradicate antibody following the start of ERT was demonstrated, as well as the long-term efficacy of liver depot therapy (Fig.3). These experiments modeled the effects of AAV2/8-LSPh-GAA administration in either: (i) the naïve Pompe subject, prior to ERT; (ii) in conjunction with ERT, either at the MED or slightly higher dose; and (iii) 4 months following ERT initiation, in a Pompe subject immunized against rhGAA.

The eradication of long-term memory antibody responses to rhGAA is perhaps the most novel aspect of this study, never achieved previously with gene therapy in Pompe disease. Previously the administration of an AAV vector to express coagulation factor VIII in dogs with hemophilia A was shown to suppress inhibitory antibodies and to establish immune tolerance.[20] Subsequently two groups showed that expression of coagulation factor IX could similarly induce immune tolerance following inhibitory antibody formation mice with hemophilia.[21,22] Similar effects have

been demonstrated following AAV vector administration in mice and dogs with hemophilia B using a high activity factor IX.[23] In Pompe disease rAAV8 vectors have been administered 2 to 3 weeks following the start of ERT to suppress anti-GAA antibody formation in GAA-KO mice.[9,11] In the current study immune tolerance induction was possible as late as Week 25. As shown in Figure 4, when ERT was administered 3 time by Week 5 and again at Week 31, GAA-KO mice formed antibodies by Week 8 that were boosted to a robust HSAT following the fourth injection (Grp 1; Fig. 4C). Mice that received two ERT treatments before the intravenous injection of AAV8-LSP-hGAA in week 5 formed no significant humoral response against GAA, even after five ERT treatments over 31 weeks (Grp 2; Fig 4C). In contrast, mice that received four ERT treatments in the first 20 weeks developed a strong humoral response against the transgene (Grp 3; Fig. 4C). Subsequently, treatment with AAV8-LSP-hGAA at Week 25 dramatically decreased the humoral response to rhGAA (Grp 3; Fig. 4C). Furthermore, an immune challenge with rhGAA at Week 31 did not induce a humoral response following treatment with AAV8-LSP-hGAA at Week 25, reflecting a robust peripheral tolerance induced by liver gene transfer. Therefore, we anticipate that AAV2/8-LSPhGAA could be administered to patients with Pompe disease in order to prevent or suppress anti-GAA antibodies and achieve efficacious GAA replacement.

A pharmacology/toxicology study with AAV2/8-LSPhGAApA was completed under good laboratory practice (GLP), which was designed to detect early or late toxicity.[16] Briefly, intravenous administration of the AAV2/8LSPhGAA vector at 1.6×10^{13}vp/kg (8-fold higher than the proposed higher dose in the proposed clinical trial described in the IND) did not cause significant short- or long-term toxicity. The vector genome was sustained in all tissues through 16-week post dosing, except for in blood, with a similar tissue tropism between males and females.[16] Administration of the vector alone, or combined with the ERT, was effective in producing significantly increased GAA activity and consequently decreased glycogen accumulation in multiple tissues, in comparison with administration of vehicle. The only

complication demonstrated was formation of low-titer anti-GAA IgG in some female mice. Anti-hGAA antibody formation has been associated with anaphylactic responses to ERT following the 3rd dose in GAA-KO mice[13], although anaphylaxis is rare in humans undergoing ERT for Pompe disease.[5] The only mortality in the current study occurred in mice treated solely with ERT (Table 1), consistent with the suppression of anti-GAA observed following administration of AAV2/8-LSPhGAApA.

Limitations of the current study include the lack of GAA-KO mice treated with long-term ERT, either alone or following AAV2/8-LSPhGAApA administration. Long-term ERT was not attempted due to the risk for hypersensitivity and mortality[8,9,13], and due to the limited supply of rhGAA available to us. Due to this limitation we did not demonstrate long-term immune tolerance induction to GAA following AAV2/8-LSPhGAApA at Week 25. However, immune tolerance induction from AAV2/8-LSPhGAApA has been persistent in this study following vector administration at Week 5, and in prior studies.[7-9]

We have confirmed that the sex-dependent lower efficacy of AAV vector in female mice also applies to Pompe disease[16,24], as suggested by other published studies in murine models.[25-27] Our pharmacology-toxicology study of AAV2/8-LSPhGAA in GAA-KO mice further supported the hypothesis that AAV vectors transduced tissues more efficiently in male mice.[16] The biodistribution analysis revealed a significantly higher number of vector genomes in the liver and heart of vector-treated male GAA-KO mice, in comparison with female mice.[16] Furthermore, biochemical correction was greater in male mice, in comparison of female mice, demonstrated by significantly lower glycogen content in the quadriceps and diaphragm of male mice, in comparison with female mice.[16] The current study showed greater biochemical efficacy and reduced immune responses in male mice with Pompe disease. Intriguingly, we demonstrated slightly higher glycogen content in the muscle of untreated male GAA-KO mice, in comparison with female mice, which correlated with lower background GAA activity in male GAA-KO mice (Fig. S2). These data indicated that in GAA-KO female mice, previously observed GAA-KO[16]

and in other mouse models[25], AAV mediated gene transfer appeared to be less efficacious, However, in non-human primates this difference was not observed[28], thus sex-related differences reported in this study are less likely to be relevant in the clinic. To this end, in a recently published clinical trial of AAV-mediated gene transfer, no evidence of differential transduction between male and female liver was observed.[29] Thus, available data suggest that efficacy of liver transduction in male and female Pompe patients will be equivalent.

Importantly, our data indicate that a minimum level of liver transduction is required to control transgene immunity in Pompe disease models. In translating of these results to the clinic, it will be therefore important to ensure sufficient liver transduction. In our study we propose a first dose that will be 2-fold higher than the MED defined by immune tolerance induction in the current study (i.e. $4x10^{11}$ vg/kg).The second cohort will receive a highly effective dose of AAV2/8-LSPhGAA, shown to suppress previously formed antibodies (Fig. 4), which is identical to the higher dose of an rAAV8 vector ($2x10^{12}$ vg/kg) administered in the hemophilia B clinical trials.[30] This higher dose was sufficient to decrease the glycogen content of skeletal muscle by >50%, demonstrating a high degree of biochemical efficacy (Fig. 4F). The selection of vector dosages that were effective in prior clinical trials further justifies the design of our proposed clinical trial. Moving forward, additional improvements to the platform could be implemented, such as the use of serotypes with reported high tropism for human hepatocytes[31,32], which seem to outperform rAAV8 in non-human primates and, possibly, in humans. However, rAAV8 vectors have performed very well in humanized mouse models suggesting that proceeding with the clinical translation of AAV2/8-LSPhGAA-mediated gene therapy for Pompe disease should be considered.[33,34]

In conclusion, these experiments define a range of doses starting from the MED, able to prevent humoral immune responses to GAA, to a therapeutic dose of AAV2/8-LSPhGAA, highly effective in eradicating a previously formed anti-GAA IgG response. In combination with the

assessment of pharmacology-toxicology of AAV2/8-LSPhGAA gene transfer for Pompe disease[16], these data support the clinical translation of liver depot gene therapy in Pompe disease. The strategy of liver-targeted gene therapy is an innovative emerging therapy with no parallel among currently approved therapies. Eventual marketing approval would represent a systemic, non-invasive gene therapy in Pompe disease. Successful clinical development would provide immunomodulatory gene therapy in Pompe disease, which would induce immune tolerance to GAA and prevent HSAT formation. This strategy could be useful in other lysosomal storage disorders, and in hemophilia, where antibody responses to therapeutic proteins frequently complicate replacement therapy.[2,35-39]

MATERIALS AND METHODS

In vivo *evaluation of AAV vector-mediated efficacy*

The AAV vector was prepared as described and administered intravenously to GAA-KO mice with a C57BL/6 background.[24,40] ERT was administered at the standard dose (20 mg/kg), injected intravenously either weekly or every other week, and diphenhydramine was injected intraperitoneally 15 minutes prior to the second and subsequent doses of ERT to prevent anaphylaxis. Age and sex-matched GAA-KO mice were housed in groups of 3 to 5, and mice from different groups were co-housed when possible. Rotarod testing was performed as described [24]. GAA activity and glycogen content were analyzed as described [24]. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Anti-GAA antibody isotypes determination

Maxisorp 96 wells plates (Thermo Fisher Scientific, Waltham, MA) were coated with Myozime protein in carbonate buffer at 4℃ overnight. A sta ndard curve of IgG isotype (Sigma Aldrich, Saint Louis, MO) was coated to the wells in seven 2-fold dilution starting from 1 µg/ml. After blocking, plasma samples diluted at 1:100 were added to plates and incubated 1 hr at 37C. Isotype-specific secondary antibodies coupled to HRP were used for detection (Southern Biotech, Birmingham,AL). Then, 3,3′,5,5′-tetramethylbenzidine substrate (BD Biosciences, San Diego, CA) was added to the wells and color development was measured at 450 and 570 nm (for background subtraction) on an Enspire plate reader (Perkin Elmer, Waltham, MA) after blocking the reaction with H2SO4.

Viral vector genome copy number analysis

Total DNA was extracted from approximately 100 mg of frozen liver tissue by using the MagNA Pure 96 DNA and viral NA small volume kit (Roche Diagnosis, Basel, Switzerland) according to manufacturer's instructions. Viral vector genome copy number (VGCN) measured by qPCR were normalized by the copies of titin gene measured in each sample. qPCR was performed on a LightCycler 480 (Roche Diagnostics, Basel, Switzerland) using SybrGreen mix (Thermo Fisher Scientific, Waltham, MA) and the following specific primers and probes: GAA forward 5'-AGATCCCCCAGACAGTGCTG-3', reverse 5'-TTCCTGCTGGCAGTGGTGCTGA-3', titin forward 5'-AAAACGAGCAGTGACGTGAGC-3', reverse 5'-TTCAGTCATGCTGCTAGCGC-3'.

RNA extraction and RT-qPCR

Total RNA was extracted from approximately 100 mg of frozen liver tissue by using the MagNA Pure 96 RNA extraction kit (Roche Diagnosis, Basel, Switzerland) according to manufacturer's instructions. Total RNA was reverse-transcribed using random hexamers and the RevertAid H minus first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). RT-qPCR was performed using SybrGreen (Thermo Fisher Scientific, Waltham, MA) with primers specific for:

CD4: forward GGTTCGGCATGACACTCT, reverse CTGACTCTCCCTCACTCTTATAG, CD8 forward ATCACTCTCATCTGCTACC, reverse GCCTTCCTGTCTGACTAG, FoxP3 forward AGGACAGACCACACTTCAT, reverse GACGCACTTGGAGCACAG, CTLA4 forward TATGTCATTGATCCAGAAC, reverse CTGTTGTAAGAGGACTTC, GAPDH forward CATGGCCTTCCGTGTTCCTA, reverse GCGGCACGTCAGATCCA. Expression levels were normalized for the level of expression of GAPDH (ΔCt) and then to the average level measured in control group (ΔΔCt). By using this method a ΔΔCt of 1 corresponds to one cycle difference in the expression levels measured by RT-PCR and this corresponds to a two-fold difference in the RNA expression.

Statistical analyses

Multiple comparisons were assessed with two-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test or with multiple t-tests using Prism software (Graphpad, La Jolla, CA). A p-value <0.05 was considered to be statistically significant.

Disclosure/Conflict of interest

DDK has developed the technology that is being used in the study. If the technology is commercially successful in the future, the developers and Duke University may benefit financially. DDK has received research/grant support from Sanofi Genzyme Corporation in the past, and rhGAA for these studies was supplied by Sanofi Genzyme.

Author Contributions

Sang-oh Han, Giuseppe Ronzitti, Benjamin Arnson, Christian Leborgne, and Songtao Li: Performed research, Analyzed data

Federico Mingozzi: Analyzed data, Contributed new reagents, Wrote paper

Dwight D. Koeberl: Analyzed data, Wrote paper

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FIGURE LEGENDS

Figure 1: Comparison of liver depot gene therapy with ERT. In a 5 week study GAA-KO mice were treated with (A) either a weekly injection of rhGAA (ERT; 20 mg/kg; n=10), or a single injection of AAV2/8-LSPhGAApA (AAV; $8x10^{11}$ vg/kg; n=10). The primary endpoints included: (B) GAA activity and (B) glycogen content in the tissues, (D) antibody formation, (E) blood GAA, and (F) heart size. GAA activity was increased and glycogen content was reduced in the heart and skeletal muscles. Mean +/- s.d. shown. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 from ANOVA.

Figure 2: AAV2/8-LSPhGAApA reduced the glycogen content of muscle, and increased the benefit from simultaneous ERT. (A) GAA-KO mice were treated with the AAV2/8-LSPhGAA vector (AAV), either with or without simultaneous ERT for 4 doses (rhGAA, 20 mg/kg every 2 weeks). Number of mice per group as follows, (-) ERT: 0 (n=8), $2x10^{10}$ (n=8), $8 x10^{10}$ (n=9), $2 x10^{11}$ (n=8); (+)ERT: 0 (n=10), $2 x10^{10}$ (n=9), $8 x10^{10}$ (n=9), $2 x10^{11}$ (n=9). Biochemical correction in GAA-KO mice was evaluated 8 weeks following gene therapy, either with (shaded bars) or without (clear bars). GAA activity and glycogen content shown for (B) heart, (C) diaphragm, and (D) quadriceps. Mean +/- s.d. shown. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 from 2 way ANOVA.

Figure 3: Liver gene transfer reduces IgG levels in a dose-dependent manner. Number of mice per group as follows, (-) ERT: 0 (n=8), $2x10^{10}$ (n=8), $8x10^{10}$ (n=9), $2x10^{11}$ (n=8); (+)ERT: 0 (n=10), $2x10^{10}$ (n=9), $8x10^{10}$ (n=9), $2x10^{11}$ (n=9). (A-D) The histograms indicate the levels of total IgG (A), IgG1 (B), IgG2b (C), and IgG2c (D), measured in plasma 9 weeks after vector injection and one week following the fourth dose of ERT. The quantification of antibody isotypes has been performed using purified mouse IgG isotypes as standard. Statistical analysis has been performed by comparison of untreated and ERT treated mice at the same vector dose

using multiple t-tests (p<0.05, (+)ERT vs (-)ERT). Nine weeks after vector injection mice were sacrificed and the VGCN was measured in the liver. IgG isotype titers where divided in three groups depending on the genome copy number measured in the liver, less than 0.1 (<0.01), between 0.01 and 1 (0.01-1) and more than 1 (>1) copies of vector genome per diploid genome. (E-H): The histograms indicate the levels of total IgG (E), IgG1 (F), IgG2b (G), and IgG2c (H) for each level of VGCN. Statistical analysis has been performed by comparison of untreated and ERT treated mice at the same vector dose using multiple t-tests (p<0.05, (+)ERT vs (-)ERT). Mean +/- s.d. shown. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 from multiple t-tests.

Figure 4: Long-term clearance of glycogen from the heart, diaphragm and skeletal muscle and preservation of neuromuscular function from higher dosage rAAV8. (A) Experimental design. GAA-KO mice were monitored for 36 weeks following administration of ERT with or without a single dose of AAV2/8-LSPhGAA ($2x10^{12}$ vg/kg) at the indicated times. (B) Histograms for anti-GAA ELISA (1:200) detecting IgG1 at the Early and Late time points. All 3 groups were challenged with rhGAA at week (Wk) 31. The Week 25 administration of rAAV8 suppressed anti-GAA from very elevated at Wk 23 to background levels by Week 36. However, the ERT only group formed higher antibodies following the immune challenge. AAV at Week 5 maintained suppression of anti-GAA. The background signal for untreated mice was <0.01 in the ELISA. (C Rotarod at Week 0 (Baseline) and Week 36. Mice were euthanized at Week 36. (D) Muscle GAA, and (E) muscle glycogen. Mean +/- s.d. shown. *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001, per 2 way ANOVA.

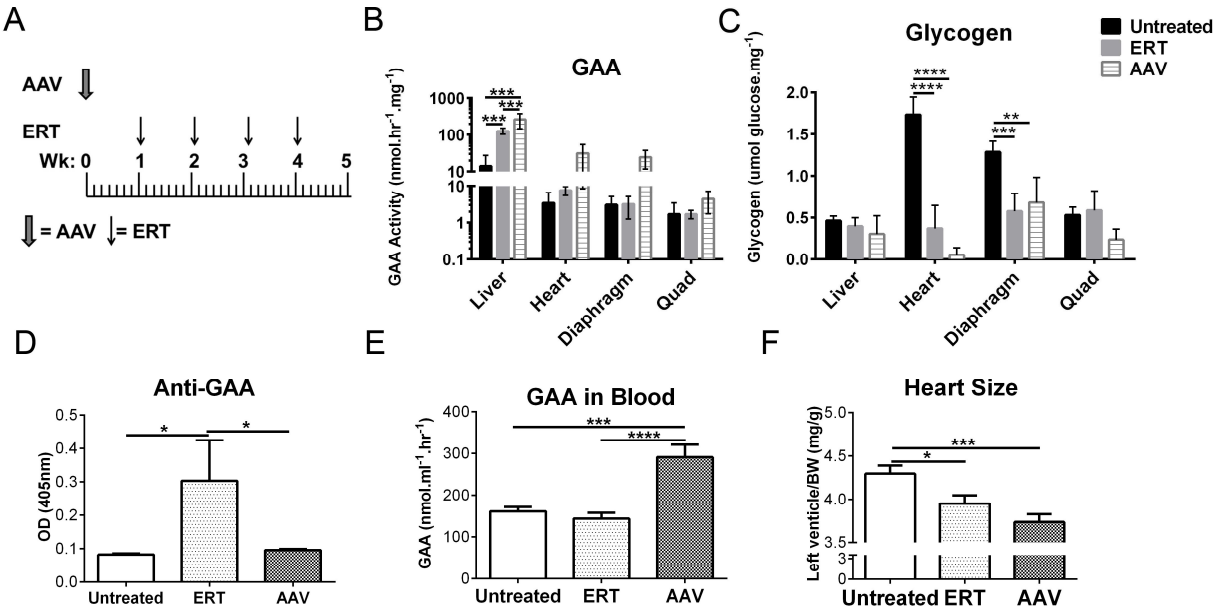
Figure 5. Liver gene transfer eradicates humoral immune response triggered by multiple rhGAA infusions. GAA-KO mice from the three groups were injected with 20 mg/kg of rhGAA and $8x10^{11}$ vg/kg of AAV8-LSP-hGAA as indicated in the experimental plan (Fig. 4A). Scatter plots show total IgG (A), IgG1 (B), and ranked IgG (C) and IgG1 (D), which was measured in plasma 36 weeks after vector injection and ranked by the degree of antibody production.

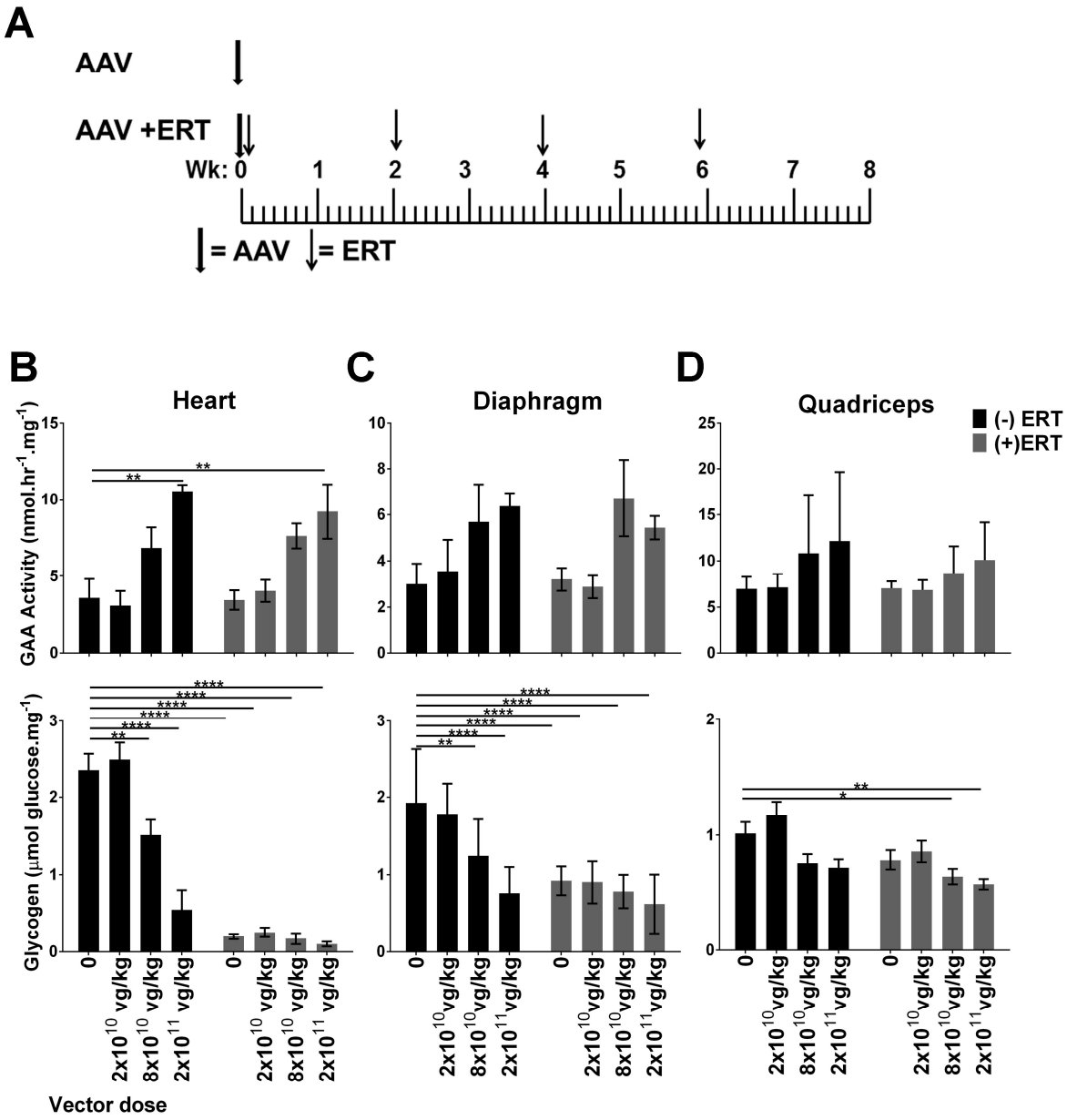
Antibodies were ranked as follows : <0.25=0, 0.25-0.5=1, 0.51-0.75=2, 0.76-1=3, and >1=4 (Supplemental Table). The quantification of antibody isotypes has been performed using purified mouse IgG isotypes as standard. Mean +/- s.d. shown. *=p<0.05, **=p<0.01 from ANOVA.

Table 1: Experimental group designation

Exp	Grp	Number animals	Study day 1	Additional dosing days		Experimental goal	Duration
				Vector[a] dose (vg/kg)	rhGAA[b] (Study day)		
1	1	10 (5M/5F)	None	0	7,14,21,28[C]	Evaluate intensive ERT	5 weeks
	2	10 (5M/5F)	AAV	$8x10^{11}$	None	Evaluate low dose AAV	
	3	8 (4M/4F)	None	0	None	Negative control	
2	1	8 (5M/3F)	None	$6x10^{9}$	None	Evaluate dose reduction to determine minimum effective dose for biochemical correction	8 weeks
	2	8 (5M/3F)	None	$2x10^{10}$	None		
	3	9 (4M/5F)	None	$8x10^{10}$	None		
	4	9 (5M/4F)	None	$2x10^{11}$	None		
	5	10 (5M/5F)	rhGAA	0	14, 28, 42	ERT only control	
	6	10 (5M/5F)	rhGAA	$6x10^{9}$	14, 28, 42	Evaluate dose reduction to determine minimum effective dose for immune tolerance induction	
	7	9 (5M/4F)	rhGAA	$2x10^{10}$	14, 28, 42		
	8	9 (5M/4F)	rhGAA	$8x10^{10}$	14, 28, 42		
	9	9 (5M/4F)	rhGAA	$2x10^{11}$	14, 28, 42		
	10	8 (5M/3F)	None	0	None	Untreated control	
3	1	11 (6M/5F)	rhGAA	0	15, 36, 220[d]	ERT to provoke antibody responses in comparison with Grp 2	36 weeks
	2	9 (5M/4F)	rhGAA	$2x10^{12}$ (Week 5)	15, 120, 134, 220	ERT followed by AAV at Week 5 to evaluate induction and duration of immune tolerance	
	3	11 (7M/4F)	rhGAA	$2x10^{11}$ (Week 25)	15, 120, 134, 220[e]	ERT to provoke antibody responses in comparison with Grp 2, followed by AAV at Week 31 to evaluate suppression of antibody responses	
	4	4 (3M/1F)	None	0	None	Untreated control	

[a]AAV2/8-LSPhGAA administered intravenously on study day 1, or at a later date as indicated.
[b]ERT consisting of injecting rhGAA intravenously (20 mg/kg). The second and subsequent ERT injections were preceded by 15 minutes with diphenhydramine through intraperitoneal injection (20 mg/kg) to prevent hypersensitivity.
[c]Two mice died: one male died following ERT at day 28, and one female died following ERT at day 7.
[d]One mouse died: one male died after day 162, unrelated to ERT.
[e]One mouse died: one male died following ERT at day 134.




A
AAV
AAV +ERT
Wk: 0 1 2 3 4 5 6 7 8
= AAV = ERT
B
Heart
C
Diaphragm
D
Quadriceps
(-) ERT
(+)ERT
GAA Activity (nmol.hr-1.mg-1)
Glycogen (µmol glucose.mg-1)
0
2x10^10 vg/kg
8x10^10 vg/kg
2x10^11 vg/kg
Vector dose