Immunomodulatory Gene Therapy Prevents Antibody Formation and Lethal Hypersensitivity Reactions in Murine Pompe Disease

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Infantile Pompe disease progresses to a lethal cardiomyopathy in absence of effective treatment. Enzyme-replacement therapy (ERT) with recombinant human acid α-glucosidase (rhGAA) has been effective in most patients with Pompe disease, but efficacy was reduced by high-titer antibody responses. Immunomodulatory gene therapy with a low dose adeno-associated virus (AAV) vector (2×10^{10} particles) containing a liver-specific regulatory cassette significantly lowered immunoglobin G (IgG), IgG1, and IgE antibodies to GAA in Pompe disease mice, when compared with mock-treated mice ($P < 0.05$). AAV-LSPhGAApA had the same effect on GAA-antibody production whether it was given prior to, following, or simultaneously with the initial GAA injection. Mice given AAV-LSPhGAApA had significantly less decrease in body temperature ($P < 0.001$) and lower anaphylactic scores ($P < 0.01$) following the GAA challenge. Mouse mast cell protease-1 (MMCP-1) followed the pattern associated with hypersensitivity reactions ($P < 0.05$). Regulatory T cells (Treg) were demonstrated to play a role in the tolerance induced by gene therapy as depletion of Treg led to an increase in GAA-specific IgG ($P < 0.001$). Treg depleted mice were challenged with GAA and had significantly stronger allergic reactions than mice given gene therapy without subsequent Treg depletion (temperature: $P < 0.01$; symptoms: $P < 0.05$). Ubiquitous GAA expression failed to prevent antibody formation. Thus, immunomodulatory gene therapy could provide adjunctive therapy in lysosomal storage disorders treated by enzyme replacement.

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INTRODUCTION

Infantile-onset glycogen storage disease type II (Pompe disease; MIM 232300) caused death early in childhood from cardiorespiratory failure related to an underlying hypertrophic cardiomyopathy, prior to the availability of enzyme-replacement therapy (ERT).[1] Pilot studies of ERT with recombinant human acid α-glucosidase (rhGAA) (purified from Chinese hamster ovary cell cultures[2] or transgenic rabbit milk[3]) resolved or improved cardiomyopathy and prolonged the survival of all subjects beyond 1 year. Pompe disease patients who lacked any residual GAA protein are deemed crossreacting immune material negative (CRIM-negative). CRIM-negative Pompe disease subjects produced very high anti-hGAA antibodies and demonstrated markedly reduced efficacy from ERT. In the first pilot study of ERT in Pompe disease using Chinese hamster ovary cell–derived recombinant hGAA, the two patients who were CRIM-negative produced higher titers of anti-hGAA antibodies than the third patient who was CRIM-positive.[2] Poor outcomes were associated with CRIM-negative status in the pivotal clinical trials that led to marketing approval for rhGAA.[4,5] CRIM-negative Pompe disease subjects in these clinical trials formed very high, sustained anti-hGAA antibodies and demonstrated markedly reduced efficacy from ERT.[2,4,5]

The antibody response to ERT in Pompe disease has been remarkably similar to inhibitory antibody formation in hemophilia.[6] Hemophilia B is similar to Pompe disease, in that CRIM-negative patients frequently mounted high-titer IgG antibody responses to protein replacement therapy with coagulation factor IX (FIX) that interfere with efficacy. Taken together, these data suggest that immune tolerance to ERT is absent in CRIM-negative patients, and that high-titer antibody formation reduced any clinical benefit from ERT.

Tolerization therapy, including administration of high-dose rhGAA with immune suppressant drugs, failed to improve the clinical response to ERT in CRIM-negative subjects. Indeed, high-dose hGAA therapy precipitated nephrotic syndrome in one of the CRIM-negative subjects, possibly related to effects of antibody complexes upon the glomerular basement membrane.[7] At present there is no successful immune modulation or tolerization protocol for patients that maintained the efficacy of ERT following the formation of anti-GAA antibodies.

The potential advantages of gene therapy over ERT have become clear in experiments with Pompe disease mice. The availability of

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novel adeno-associated virus (AAV) serotypes, including AAV8, advanced gene therapy by improving the tropism of vectors for target tissues.[8] AAV2 vectors pseudotyped with AAV8 (AAV2/8) delivered genes to the liver ~100-fold more efficiently in mice, including GAA-knockout (KO) mice, in comparison with traditional AAV2 vectors.[8,9]

Liver-restricted expression of GAA with an AAV vector prevented the formation of anti-hGAA antibodies in GAA-KO mice. A single administration of a low dose AAV2/8 vector containing a liver-specific regulatory cassette substantially corrected glycogen storage in the diaphragm and heart of GAA-KO mice [3×10^{10} vector particles (vp), equivalent to 1×10^{12} vp/kg], whereas an even lower dose prevented anti-GAA antibody formation without achieving biochemical correction.[10] Another AAV vector containing a liver-specific regulatory cassette expressed high-level hGAA in the liver of adult GAA-KO mice for over 12 weeks without provoking a detected anti-hGAA IgG response.[11] Increasing plasma hGAA was detected between 1 and 14 days and sustained for >12 weeks following AAV-LSPhGAApA administration.[11] The aforementioned AAV vectors contained a liver-specific regulatory cassette that drove therapeutically relevant coagulation FIX expression and diminished antibody responses in hemophilia B mice and dogs.[12,13] These data suggested that liver-restricted, high-level expression of hGAA induced immune tolerance in Pompe disease mice, similarly to the effect of liver-specific expression of therapeutic proteins in hemophilia mice[12,14] and in Fabry disease mice.[15]

Like CRIM-negative patients with Pompe disease, GAA-KO mice lack immune tolerance to hGAA and ERT has no efficacy, even provoking fatal anaphylaxis.[16] A strategy for immunomodulatory gene therapy was developed in GAA-KO mice by administering a low number of AAV-LSPhGAApA particles prior to the initiation of ERT, thereby inducing immune tolerance to rhGAA and enhancing the efficacy of ERT.[17] This approach to immunomodulatory gene therapy was apparently limited by the need to administer the vector prior to initiation of ERT, which seemed to be required to prevent antibody formation. Currently, we have evaluated the kinetics and mechanism for immunomodulatory gene therapy in GAA-KO mice to better understand the potential clinical application of this strategy.

RESULTS

Vector-mediated immune modulation prevented mortality in Pompe disease mice

The tolerogenic AAV vector (AAV-LSPhGAApA) previously induced immune tolerance to hGAA, by preventing the anti-GAA antibody response observed in naive GAA-KO mice.[17] The timing of AAV vector administration relative to the immune challenge with hGAA was further evaluated in adult GAA-KO mice (**Table 1**; **Figure 1a**). AAV-LSPhGAApA administration (2×10^{10} vp intravenously) significantly prolonged survival in groups (G) of mice, if administered either prior to (G1; $P = 0.05$) or following (G2; $P = 0.05$) initial rhGAA injection (**Figure 1b**). AAV-LSPhGAApA administration enhanced the efficacy of ERT, as reflected by the increased time that vector-treated GAA-KO mice could run on the Rotarod (**Figure 1c**; G1-3). Decreased Rotarod time indicates progressive loss of muscle function in GAA-KO mice, which can be prevented by reducing the glycogen content of striated muscle.[11]

Table 1 Immune challenges prior to ERT

Group	Week 0	Week 3
G1	AAV-LSPhGAApA	rhGAA
G2	rhGAA	AAV-LSPhGAApA
G3	AAV-LSPhGAApA + rhGAA	rhGAA
G4	rhGAA	rhGAA
G5	PBS	rhGAA
G6	AAV-CBhGAApA	PBS
G7[a]	PBS	PBS

Abbreviations: AAV, adeno-associated virus; ERT, enzyme-replacement therapy; PBS, phosphate-buffered saline; rhGAA, recombinant human acid α-glucosidase. Week 6, rhGAA injection. Week 12–22, rhGAA (20 mg/kg) every other week.
[a]No ERT was administered to group G7 providing mock-treated GAA-KO mice.

Urinary Hex_4, a biomarker that was decreased in correlation with biochemical correction in Pompe disease mice, was reduced following AAV-LSPhGAApA administration, in comparison with GAA-KO mice that received rhGAA injections only (**Figure 1d**; G1-3). The negative impact of pre-existing anti-GAA antibodies was demonstrated by the persistent elevations of urinary Hex_4 following antibody formation. GAA-KO mice that were immunized with two injections of rhGAA demonstrated elevated urinary Hex_4 at week 10, in comparison with a group of phosphate-buffered saline–injected GAA-KO mice that had not yet formed anti-GAA antibodies (**Figures 1d** and **2a**; G5 versus G4).

Formation of anti-GAA antibodies associated with hypersensitivity reactions to rhGAA challenge

The formation of anti-GAA antibodies occurred uniformly, if mice were not treated with AAV-LSPhGAApA (**Figure 2a**, G4 and G5). In contrast, tolerogenic AAV vector administration suppressed IgG titers, even when vector administration followed the initial rhGAA injection by 3 weeks (**Figure 2b**, G2). IgG titers reached 1:8,000 in response to four injections of rhGAA in mock-treated mice (G4), whereas groups of vector-treated mice had significantly reduced titers at week 10 (**Figure 2b**). The primary antibody response was IgG1 (**Figure 2c**), although IgE was significantly elevated in G4, in comparison with groups treated with AAV-LSPhGAApA (**Figure 2d**). One-way analysis of variance confirmed significant differences in the antibody response at 6 weeks ($P = 0.0006$) in vector-treated mice (G1, G2, G3), in comparison with mice treated with ERT only (G4, G5).

Decreased body temperature and increased signs of hypersensitivity, quantified by an allergy score, have correlated with hypersensitivity reactions in mice.[18] Hypersensitivity was demonstrated by decreased body temperature (**Figure 3a**; G4 and G5) and allergic reactions in mice that were not immune tolerant to rhGAA. The nontolerant mice became immobile and died within 30 minutes of the rhGAA challenge (right panel, **Figure 3b**). The allergy score[18] was adopted to quantify symptoms ranging from eye edema (score = 2) to death (score = 5) in nonvector-treated mice (**Figure 3c**, G4 and G5). Simultaneous administration of the vector and rhGAA (G3) failed to completely prevent changes in both body temperature (**Figure 3a**) and allergy scores (**Figure 3c**),

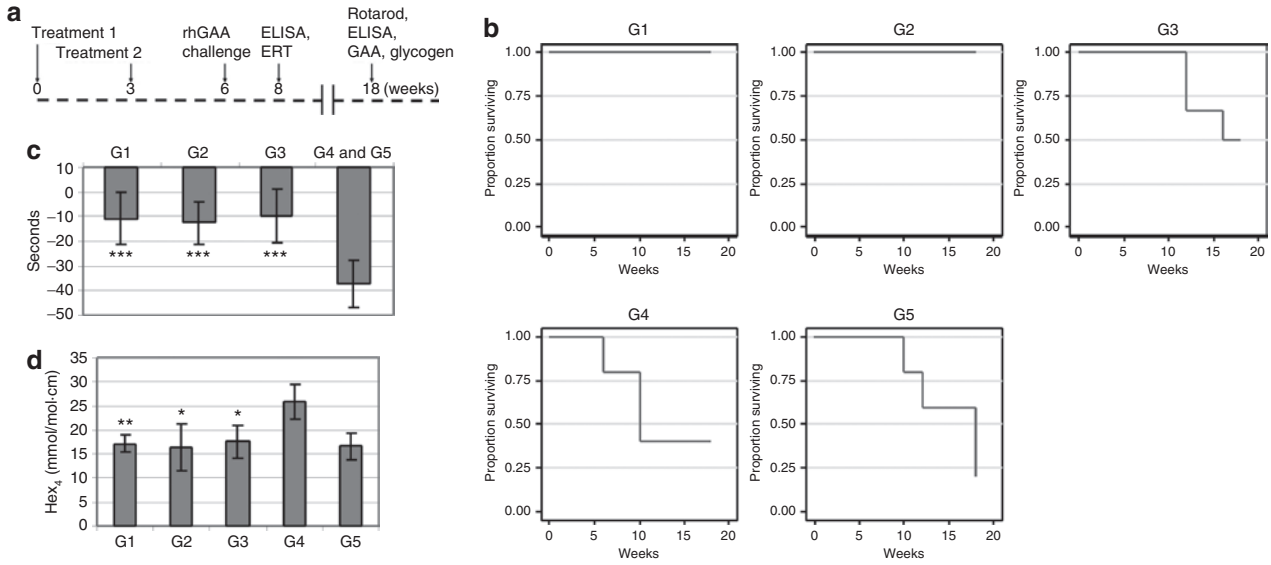

Figure 1 Enhanced efficacy and prevention of mortality by AAV vector administration preceding or following initiation of ERT. (**a**) Timeline for experiment. (**b**) Survival of GAA-KO mice either following (G1), prior to (G2), or simultaneously with (G3) administration of AAV-LSPhGAApA; in comparison with no vector administration (G4, G5) or administration of an immunogenic vector (AAV-CBhGAApA; G6), and during subsequent biweekly rhGAA administration (20 mg/kg) to simulate ERT. Each group was initially comprised 3-month-old GAA-KO mice ($n = 5$). (**c**) Rotarod times, indicating decreased muscle function between weeks 0 and 18. G4 and G5 were combined due to mortality over the course of the study. G1, G2, G4 and G5, $n = 5$; G3, $n = 4$. (**d**) Urinary Hex_4 at week 10. G1 and G2, $n = 5$; G3, G4, and G5, $n = 4$. Mean ± SD shown. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, indicating comparisons either with G4 or with G4 and G5 combined (homoscedastic *t*-test). AAV, adeno-associated virus; ELISA, enzyme-linked immunosorbent assay; ERT, enzyme-replacement therapy; G, group; GAA, α-glucosidase; KO, knockout.

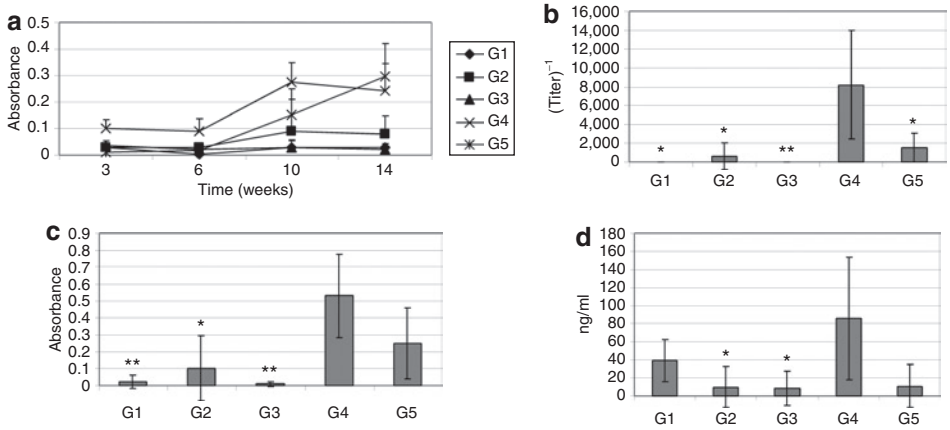

Figure 2 Antibody responses to hGAA. (**a**) IgG responses detected by anti-GAA ELISA. (**b**) Inverse titer of anti-hGAA IgG at week 10. (**c**) IgG1 responses at week 10. (**d**) IgE responses at week 10. G1, G2, G3, and G5, $n = 5$; G4, $n = 4$ (mean ± SD shown). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, indicated for each group in comparison with G4 (homoscedastic *t*-test). ELISA, enzyme-linked immunosorbent assay; G, group; hGAA, human acid α-glucosidase; IgG, immunoglonin G.

in comparison with untreated GAA-KO mice (G7); however, the changes in body temperature and allergy scores were significantly reduced in the simultaneous administration group (G3), in comparison with the rhGAA only groups (G4, G5).

Hypersensitivity reactions were associated with elevated mouse mast cell protease-1 (MMCP-1) in nonvector-treated GAA-KO mice, in comparison with untreated GAA-KO mice (**Figure 3d**; G4 and G5 versus G7). MMCP-1 was previously increased markedly during anaphylaxis in mice sensitized to a strong antigen, ovalbumin, in association with elevated IgG and IgE.[19,20] Cytokines associated with hypersensitivity, including interferon-γ, interleukin (IL)-4, and IL-5,[18] were not elevated in mice exhibiting hypersensitivity reactions (data not shown). Similarly, cytokines associated with suppression, including IL-10 and transforming growth factor-β, were not elevated in vector-treated mice immediately following rhGAA administration (data not shown).[21] One-way analysis of variance confirmed significant differences in the allergy score (<0.0001), body temperature (<0.0001), and MMCP-1 levels (<0.0001) in AAV-LSPhGAApA-treated mice (G1, G2, G3) following the immune challenge with rhGAA, in comparison with phosphate-buffered saline-treated mice (G4, G5). In contrast, administration of an immunogenic AAV vector (2×10^{10} vp) containing a ubiquitously active regulatory cassette (AAV-CBhGAApA)[11] provoked lethal

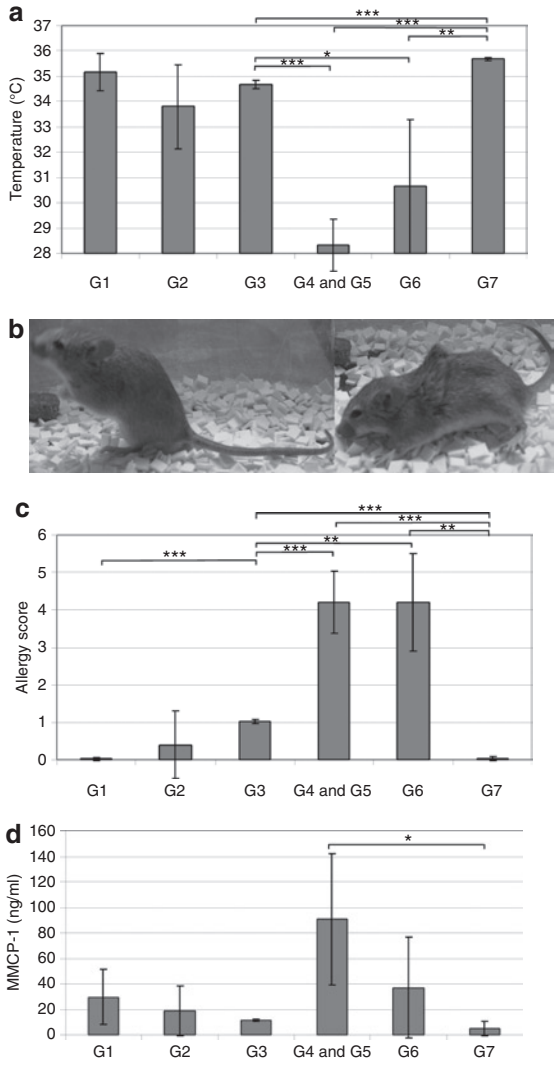

Figure 3 Hypersensitivity reactions associated with elevated MMCP-1 in nonvector-treated GAA-KO mice. (**a**) Temperature measured 30 minutes following rhGAA^{+} adjuvant challenge. G1 and G2, $n = 5$; G4 and G5 and G6 and G7, $n = 4$. (**b**) GAA-KO mouse prior to and 10 minutes following rhGAA administration, illustrating an allergy score of four. (**c**) Allergy score, G1, G2, and G6, $n = 5$; G3, $n = 3$; G4 and G5 and G7, $n = 4$. (**d**) MMCP-1 levels, each measured 30 minutes following rhGAA^{+} adjuvant challenge. G1 and G2, $n = 5$; G4 and G5 and G6 and G7, $n = 4$. Results for G4 and G5 were combined (G4 and G5), due to mortality. Mean ± SD shown. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (homoscedastic *t*-test). Lines overhead indicate the values being compared, either with G3 or with untreated GAA-KO mice (G7). KO, knockout; MMCP-1, mouse mast cell protease-1; rhGAA, recombinant human acid α-glucosidase.

hypersensitivity reactions associated with decreased body temperature and increased allergy scores (**Figure 3a,c**; G6). When AAV-CBhGAApA was administered to 3-month-old wild-type mice to sensitize GAA sufficient animals to rhGAA, anti-GAA IgG1 was detected in vector-treated wild-type mice 6 weeks later (data not shown).

A lack of efficacy from ERT was anticipated in GAA-KO mice that exhibited lethal hypersensitivity responses (**Figure 3**). The efficacy of ERT was evaluated by GAA analysis and glycogen quantification of the heart and skeletal muscles after ERT (**Table 1**). GAA activity was elevated in the muscles of all vector-treated

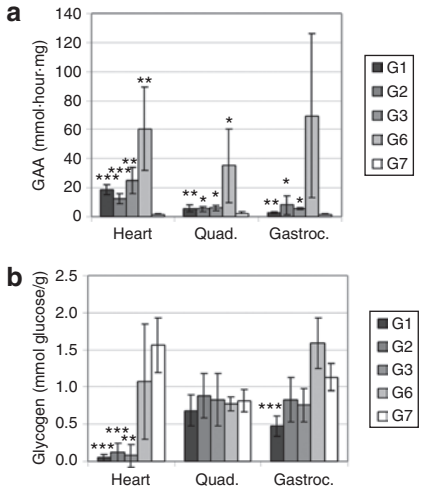

Figure 4 Efficacy of ERT following induction of immune tolerance in GAA-KO mice. (**a**) GAA activity, and (**b**) glycogen content of heart and skeletal muscles in GAA-KO mice (**Table 1**). G1 and G2, $n = 5$; G3 and G6, $n = 3$; G7, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (homoscedastic *t*-test), in comparison with untreated GAA-KO mice (G7). ERT, enzyme-replacement therapy; G, group; GAA, α-glucosidase; Gastroc, gastrocnemius; KO, knockout; Quad, quadriceps.

Table 2 Vector dosage titration

Group	Week 0	Week 6
G1	AAV-LSPhGAApA (6×10^{10} vp/mouse) + rhGAA (20 mg/kg)	rhGAA (20 mg/kg)
G2	AAV-LSPhGAApA (6×10^{9} vp/mouse) + rhGAA (20 mg/kg)	rhGAA (20 mg/kg)
G3	rhGAA (20 mg/kg)	rhGAA (20 mg/kg)
G4	PBS	rhGAA (20 mg/kg)

Abbreviations: AAV, adeno-associated virus; PBS, phosphate-buffered saline; rhGAA, recombinant human acid α-glucosidase; vp, vector particles.
Week 6, rhGAA injection, followed by allergy scoring, temperature measurement, serum and tissue collection.

mice (**Figure 4a**; G1, G2, G3, and G6). However, the relevance of lacking immune tolerance was demonstrated in GAA-KO mice that received the immunogenic vector, which had elevated glycogen content despite the presence of supraphysiologic GAA activity (**Figure 4b**; G6). One-way analysis of variance revealed that the glycogen content in the heart was significantly reduced only by AAV-LSPhGAApA administration ($P < 0.0001$), in comparison with ERT only. As reported, the efficacy of ERT was reduced in skeletal muscle, in comparison with the heart, even following the induction of immune tolerance.[17] Only when AAV-LSPhGAApA administration preceded rhGAA injection by 3 weeks did ERT significantly reduce glycogen content in the gastrocnemius, in comparison with sham-treated GAA-KO mice (**Figure 4b**; G1).

The vector particle number should be as low as possible to enhance safety from immunomodulatory gene therapy, while maintaining efficacy. The number of vector particles administered simultaneously with rhGAA, as in G3 above, was varied threefold higher and lower to model the likely scenario of immunomodulatory gene therapy and ERT starting together (**Table 2**). GAA-KO mice were challenged with rhGAA (20 mg/kg) 8 weeks following the initial vector and rhGAA administration and mortality was

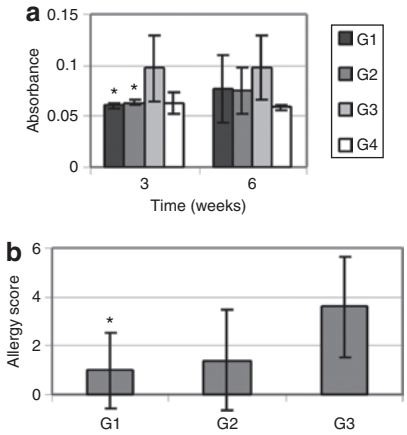

Figure 5 Effect of varying vector dose at the time of initiation of ERT upon prevention of hypersensitivity and mortality. (**a**) ELISA detection of anti-hGAA IgG1. (**b**) Allergy score 30 minutes following rhGAA (20 mg/kg) administration at 8 weeks. G1, $n = 6$; other groups, $n = 5$. ELISA, enzyme-linked immunosorbent assay; ERT, enzyme-replacement therapy; IgG, immunoglobin G; rhGAA, recombinant human acid α-glucosidase.

significantly decreased by the higher dose (6×10^{10} vp/mouse; $P < 0.05$), in comparison with mice treated with rhGAA alone (G1 versus G3, **Figure 5a**). The decrease in mortality approached significance following administration of the lowest dose (6×10^{9} vp/mouse; G2; $P = 0.07$); moreover, the lowest and highest vector doses had a comparable effect, because only one out five mice developed a lethal hypersensitivity reaction in G2 versus one out of six mice in G1 (**Figure 5a**). Antibody formation was significantly suppressed by vector administration at either dose by 3 weeks, but not at 6 weeks (**Figure 5b**), which was unexplained. The allergy score was similarly significantly reduced only by the highest vector dose (**Figure 5c**; G1), as was the change in body temperature following rhGAA administration (data not shown). Therefore, a threefold reduced vector dose significantly suppressed antibody formation only at 3 weeks following vector administration, indicating a waning effect of the lower vector dose.

Regulatory T cell–mediated immune tolerance to rhGAA and desensitization in GAA-KO mice

The role of regulatory T cell (Treg) in immune tolerance has recently been elucidated and is currently an area of intense investigation.[22–24] The depletion of Treg with anti-CD25 mouse monoclonal PC61 has been characterized as a strategy to investigate the role of Treg in immune tolerance.[22,23] The effect of depleting Treg is to increase the proliferation of T cells, thereby preventing immune tolerance. Treatment with PC61, monoclonal anti-CD25, rapidly reduces the overall number of $CD4^{+}CD25^{+}$ T cells (including Treg) by approximately fourfold, acting more effectively than denileukin diftitox or cyclophosphamide (**Figure 6a**). Administration of PC61 on day 3 following AAV-LSPhGAApA led to a subsequent rise in anti-GAA IgG following an immune challenge with rhGAA, in comparison with GAA-KO mice that received AAV-LSPhGAApA only (**Figure 6b**). Allergy scores and body temperature were significantly altered in PC61-treated mice following the immune challenge (**Figure 6c**). Thus, Treg contributed to immune tolerance in Pompe disease mice following immunomodulatory gene therapy.

DISCUSSION

The benefit of immune tolerance to rhGAA has been demonstrated in GAA-KO mice, a strain that otherwise developed immunity to hGAA and failed to respond to ERT.[16] These studies demonstrated that immune tolerance to hGAA was achieved for >18 weeks in adult, immunocompetent GAA-KO mice, through a single administration of a subtherapeutic number of AAV vector particles containing a liver-specific transgene encoding hGAA. Immune tolerance was demonstrated through an immune challenge with rhGAA, when only AAV-LSPhGAApA-treated Pompe disease mice failed to produce interfering antibodies and demonstrated efficacious responses to ERT. The immune tolerance in vector-treated GAA-KO mice contrasted markedly with the robust immune responses observed in mock-treated Pompe disease mice, because the latter groups mounted an antibody response against rhGAA that was associated with lethal hypersensitivity reactions to ERT.[16] Fortunately, Pompe disease patients who mount antibody responses experience milder infusion reactions to ERT that can be managed by slowing the infusion rate and administering antihistamines and anti-inflammatory drugs.[25] However, the clinical significance of high-titer anti-GAA antibody formation has been increasingly recognized due to decreased long-term efficacy in that subset of patients.[26] Immune tolerance to rhGAA clearly depended upon liver-specific transgene expression, which was emphasized by the lethal hypersensitivity reactions observed in GAA-KO mice treated with the immunogenic vector containing the ubiquitously active CB regulatory cassette.

The term desensitization indicates the suppression of Th2-type antibody levels in association with reduced anaphylactic reactions,[18] and immunomodulatory gene therapy achieved desensitization in these experiments with Pompe disease mice. Desensitization of GAA-KO mice following anti-GAA antibody formation reduced mortality and antibody formation. Even when AAV-LSPhGAApA was administered 3 weeks after the initial injection of rhGAA, survival was increased and anti-GAA antibodies were suppressed. The ability to suppress antibody titers and to enhance the efficacy of ERT could have important implications for CRIM-negative Pompe disease patients, in whom a lack of sustained efficacy has been associated with high-titer antibodies.[2]

Hypersensitivity reactions in GAA-KO mice were somewhat similar to infusion reactions seen in patients with Pompe disease undergoing ERT, which were allergic and included rash, fever, urticaria, and blood oxygen desaturation.[5] However, it is important to note that the immune response to rhGAA is less severe in Pompe disease patients, although it prevents long-term efficacy from ERT. CRIM-negative Pompe disease patients in the pivotal trials of ERT with rhGAA were ventilator-dependent and had much higher mortality than CRIM-positive patients (P.S. Kishnani, P.C. Goldenberg, S.L. DeArmey, J. Heller, D. Benjamin, S. Young *et al.*, manuscript submitted).[2,4,5]

The mechanism for desensitization to rhGAA involves Treg, because depleting Treg increased antibody formation in response to hGAA. MMCP-1 was implicated as a mediator of hypersensitivity reactions in GAA-KO mice, consistent with its role in allergic reactions.[19,20] IgG1 and IgE antibodies against rhGAA were elevated in Pompe disease mice that developed hypersensitivity reactions, and both types of immunoglobulins have been associated with hypersensitivity responses and elevations of MMCP-1 in mice.[20]

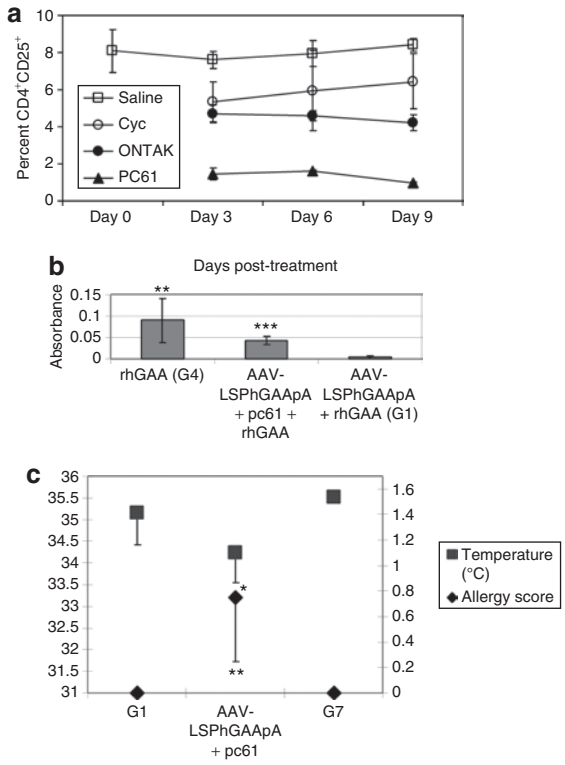

Figure 6 Depletion of Treg with PC61. (**a**) C57/BL6 Tg-CEA mice were treated with the agents listed (saline, 2 mg cyc, 18 mcg/kg ONTAK, or 0.25-mg anti-mouse CD25 depleting antibody PC6.1). Three mice per treatment group were sacrificed on days 0, 3, 6, and 9 and cells isolated from their lymph nodes were analyzed for $CD4^+CD25^+$ expression. ONTAK is recombinant IL-2 linked to diphtheria toxin. (**b**) Anti-CD25 antibody, PC61 (100 µg), was administered to a group of GAA-KO mice 6 weeks following AAV-LSPhGAApA administration (AAV-LSPhGAApA$^+$ PC61, n = 4). An immune challenge with hGAA plus adjuvant was administered 4 days later as described.[17] ELISA done 2 weeks after the immune challenge showed that IgG was significantly increased by PC61, in comparison with GAA-KO mice treated with AAV-LSPhGAApA alone (G1, n = 5). GAA-KO mice that were not treated with AAV-LSPhGAApA prior to the immune challenge formed high-titer anti-GAA antibodies (G4; n = 10). Untreated GAA-KO formed no antibodies (data not shown). (**c**) Changes in body temperature (vertical axis, left) and allergy scores (vertical axis, right) 30 minutes following rhGAA administration 8 weeks following PC61 administration. *P < 0.05, **P < 0.01, and ***P < 0.001, in comparison with AAV-LSPhGAApA alone (G1). AAV, adeno-associated virus; cyc, cyclophosphamide; ELISA, enzyme-linked immunosorbent assay; KO, knockout; ONTAK, denileukin diftitox; PBS, phosphate-buffered saline; rhGAA, recombinant human acid α-glucosidase; Treg, regulatory T cells.

The mechanisms for inducing immune tolerance to a peptide antigen have been investigated, and provide a framework for understanding of how liver-specific transgene expression might induce immune tolerance. During the induction of immune tolerance through oral or nasal administration, a shift from Th1 and Th2 responses to Th3 and Tr1 responses occurs, resulting in decreased cytotoxic T lymphocyte and antibody responses.[21] The secretion of IL-10 and transforming growth factor-β correlates with these changes and stimulates Treg involved in suppression. Treg interact with antigen-presenting cells to reduce $CD4^+$ helper T cells, thus suppressing antibody production by B cells and impairing cytotoxic T cell responses. These mechanisms for inducing tolerance have been demonstrated in a mouse model for hemophilia B. Following nasal administration of a FIX-derived peptide antigen, IL-10, and transforming growth factor-β levels increased and Treg were shown to suppress antibody formation.[27] Isolation of $CD4^+CD25^+$ Tregs from tolerant donor mice and transfer to naive recipients resulted in a transfer of immune tolerance. In contrast, we demonstrated no secretion of IL-10 following immunomodulatory gene therapy and subsequent immune challenge with rhGAA in Pompe disease mice. It is possible that Treg directly suppressed antibody production by B cells in our experiments.[28] Other experiments in hemophilia B mice revealed that hepatic expression of hFIX induced Treg in lymphoid organs that suppressed antibody formation following adoptive transfer, and depletion of Treg with PC61 prevented immune tolerance.[29] These results further supported the role of Treg in the induction of immune tolerance to human FIX,[28] which has now been implicated in the maintenance of immune tolerance to rhGAA in Pompe disease mice by depletion of Treg with an anti-CD25 antibody.

Several criteria seem to dictate whether transgene expression in the liver is sufficient to induce immune tolerance in KO mouse models. The level of transgene expression must be high and it must be mostly restricted to the liver. The obvious example of violating the requirement for liver-specific expression was amply demonstrated by driving high-level, ubiquitously active hGAA expression with a regulatory cassette containing the cytomegalovirus enhancer in AAV-CBhGAApA. The cytomegalovirus enhancer-containing vector provoked cellular and humoral immune responses that eliminated hGAA expression within 2 weeks.[11] The choice of AAV8-pseudotyped vector increases hepatic expression and further suppresses antibody responses in Pompe disease[11] and hemophila B mice,[30] in comparison with AAV2 vectors that transduced the liver less efficiently. The latter study evaluated a threefold lower dose than the lowest number of vector particles currently used to suppress antibody formation.

Previously liver-specific expression had been implicated in preventing the antibody responses to hGAA in GAA-KO mice, and the associated elevation of hGAA levels in the blood and tissues correlated with the correction of glycogen storage in the striated muscles.[11,10,31] The biochemical correction of striated muscle was associated with functional improvement in the Rotarod test[11,10,31] and in the wire hanging test.[31] However, the vector dose requirements were apparently higher than in the current study. The lowest number of vector particles to establish immune tolerance to hGAA was $<1 \times 10^{11}$ and $>1 \times 10^{10}$ vp, when an AAV2/8 vector containing a different liver-specific regulatory cassette was administered prior to an immune challenge with rhGAA and adjuvant.[31] The lowest tolerogenic dose of AAV-LSPhGAApA was 7×10^9 vp, and the limiting dose for the establishment of immune tolerance has yet to be established.

The significance of antibody responses to ERT in lysosomal storage disorders varies somewhat, although CRIM-negative subjects often respond poorly. A retrospective analysis of CRIM-negative Pompe disease patients clearly demonstrated an attenuated response to enzyme in all outcome measures compared to CRIM-positive patients: significantly decreased survival, invasive ventilation free survival, less improvement in cardiac response, and a regression of motor milestones (P.S. Kishnani, P.C. Goldenberg,

S.L. DeArmey, J. Heller, D. Benjamin, S. Young *et al.*, unpublished results).[2,4,5] In Fabry disease hemizygous male patients mount an antibody response to ERT much more frequently than females, presumably because males are more often CRIM-negative.[32,33] Although, the clinical relevance of antibody formation in Fabry disease remains to be determined, recently the presence of high-titer IgG was associated with increased storage of globotriasylceramide in capillary endothelial cells.[34] Antibody formation in dogs with mucopolysaccharidosis type I reduced the efficacy of ERT.[35] Mucopolysaccharidosis type I patients excreted higher quantities of urinary glycosaminoglycans in association with high-titer antibody formation in one clinical trial of ERT, and antibody formation was associated with reduced efficacy in another trial.[36,37] Thus, antibody responses tend to negatively impact the efficacy of ERT in lysosomal storage disorders, and immunomodulatory therapies should be developed at least for CRIM-negative patients.

An immunomodulatory gene therapy strategy could be an important adjunct to ERT in CRIM-negative Pompe disease patients. The efficacy of ERT would be enhanced by preventing or suppressing antibody responses, and safety would be enhanced by the low number of vector particles needed to induce immune tolerance.[17] The advantage of suppressing antibody formation after the initiation of ERT would be to allow early treatment prior to the determination of CRIM status. This strategy could also be utilized in other disorders where antibodies interfere with protein therapy, including hemophilia, lysosomal storage disorders, and type 1 diabetes. Furthermore, hypersensitivity reactions caused by an identified peptide antigen, such as peanut allergies, could potentially be treated with immunomodulatory gene therapy.[18]

MATERIALS AND METHODS

Preparation of AAV2/8 vectors. Briefly, 293 cells were transfected with the pAAV-LSPhGAApA vector or pAAV-CBhGAApA vector plasmid,[11] the AAV packaging plasmid p5E18-VD 2/8 (ref. 8) (courtesy of Dr James M. Wilson, University of Pennsylvania, Philadelphia, PA), and pAdHelper (Stratagene, La Jolla, CA). The liver-specific promoter regulatory cassette (subcloned from pAV-LSP-cFIX, courtesy of Dr Inder Verma, Salk Institute, La Jolla, CA; sequence available upon request) contains a thyroid hormone-binding globulin promoter sequence downstream from two copies of a α1-microglobulin/bikunin enhancer sequence,[38] and previously achieved long-term efficacy in hemophilia B mice within an AAV vector encoding coagulation FIX.[12] Cell lysate was harvested 48 hours following infection and freeze-thawed three times, and isolated by sucrose cushion pelleting followed by two cesium chloride gradient centrifugation steps. AAV stocks were dialyzed against three changes of Hanks buffer, and aliquots were stored at −80 °C. The number of vector DNA containing-particles was determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the National Institutes of Health.

In vivo *analysis of AAV vector.* The AAV2/8 vector stocks were administered intravenously (via the retro-orbital sinus) in 3-month-old GAA-KO mice.[39] At the indicated time points postinjection, plasma or tissue samples were obtained and processed as described below. Rotarod testing was performed as described.[9] GAA activity and glycogen content were analyzed as described.[40] ERT was modeled in GAA-KO mice by retro-orbital injection of rhGAA (5 mg/ml; supplied by Genzyme, Framingham, MA) over ~15 seconds.

All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee–approved guidelines.

Antibody quantification. The enzyme-linked immunosorbent assay for IgG and IgG subtypes was performed as described.[41] Enzyme-linked immunosorbent assay for IgE was performed as described previously.[42] Briefly, plates were coated with 5 μg/ml rhGAA in carbonate–bicarbonate buffer at pH 9.6. Plasma samples were pretreated with protein G agarose beads (Pierce Biotechnology, Rockford, IL) to remove IgG and used at a final dilution of 1:20. Detection of rhGAA-bound IgE was performed with sheep anti-mouse IgE (0.5 μg/ml; Binding Site, Birmingham, UK), followed by biotinylated donkey anti-sheep IgG (0.5 μg/ml; Accurate Chemical, Westbury, NY) and neutravidin-horseradish peroxidase (0.2 μg/ml; Pierce). The horseradish peroxidase activity was measured with color development of 3,3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithersburg, MD). All samples yielded absorbance values that were within the linear range of the assay at this dilution.

In vivo *depletion of Treg.* C57/BL6 Tg-CEA mice were injected intraperitoneally on day 0 with 2 mg cyclophosphamide, 18 μg/kg denileukin diftitox (Eisai, Woodcliff Lake, NJ), 0.25 mg PC6.1 (BD Biosciences, San Jose, CA), or saline only in total volume of 250 μl. On day 0 prior to treatment and on days 3, 6, 9 following treatment, three mice from each group were killed, lymph nodes removed, and immune cells isolated. The freshly isolated cells were then stained for 30 minutes with CD25-FITC, CD3-PerCP, and CD4-APC and analyzed by multiparameter flow cytometry using a fluorescence-activated cell sorting Calibur (BD Biosciences). The total percent $CD4^+CD25^+$ cells were determined by gating on lymphocytes using forward and side scatter and $CD3^+$ T cells.

Statistical analyses. Multiple comparisons were performed with a one-way analysis of variance, and individual comparisons between groups were performed with a homoscedastic Student's *t*-test as noted. A P value of ≤ 0.05 indicated a significant difference between the observed values for each group of GAA-KO mice following AAV vector administration and the control group(s) of phosphate-buffered saline-injected GAA-KO mice.

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