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Deficiency in MyD88 Signaling Results in Decreased Antibody Responses to an Adeno-Associated Virus Vector in Murine Pompe Disease

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Abstract

We have previously shown that antibody and T cell responses limit the efficacy of an adeno-associated virus (AAV) pseudotype 8 (2/8) vector containing the universally active cytomegalovirus enhancer/chicken β-actin regulatory cassette (AAV2/8-CBhGAA) in treating murine Pompe disease. However, the innate immune responses to AAV2/8-CBhGAA are largely unknown. In this study, we investigated acute immune responses to AAV2/8-CBhGAA and the role of MyD88/TRIF signaling pathway in shaping adaptive immune responses to this vector. We showed here that a small and transient increase in CXCL-1 and IL-1β expression in livers of acid-α-glucosidase knockout (GAAKO) mice 6 h following injection with AAV2/8-CBhGAA. There was a robust antibody response to GAA in wild-type mice injected with this vector. In contrast, the anti-GAA IgG1 response was diminished in MyD88KO mice, and showed a trend toward a decrease in TRIFKO mice. In addition, the vector genome and GAA activity were significantly higher in MyD88KO livers compared with wild-type livers, suggesting reduced cytotoxic T cell responses. Importantly, elevated $CD4^+$ T cells were detected by immunohistochemistry in MyD88KO livers. When adoptively transferred to wild-type mice, these $CD4^+$ T cells have an ability to suppress antibody responses against AAV2/8-CBhGAA and to prevent further immunization against rhGAA. Our study suggests that the MyD88 deficiency leads to the suppression of deleterious immune responses to AAV2/8-CBhGAA, which has implications for gene therapy in Pompe disease.

Key words: AAV vectors; acid-alpha glucosidase; gene transfer; glycogen storage disease; immunology

Introduction

Gene therapy has great potential for the potentially curative treatment of genetic diseases involving muscle, including metabolic myopathies such as Pompe disease and the muscular dystrophies. Indeed, gene therapy with adeno-associated virus (AAV) vectors has yielded proof-of-concept in many preclinical experiments and advanced to clinical trials in Duchenne muscular dystrophy (DMD).[1] However, unanticipated T cell responses directed against AAV vector-mediated expression of dystrophin have complicated the clinical trial of muscle-targeted gene therapy in DMD.[2] Similarly, ubiquitous expression of acid-α-glucosidase (GAA) with an AAV vector provoked cytotoxic T lymphocyte (CTL) responses in GAA knockout (KO) mice, which eliminated transgene expression within weeks.[3,4] Thus, immune responses directed against the transgene must be addressed before clinical translation of gene therapy in the inherited diseases of muscle.

Widespread transgene expression will be needed to correct the neuromuscular involvement of Pompe disease, which includes the striated muscle, smooth muscle, motor neurons, and central nervous system.[5] Expression of GAA with an AAV vector containing the ubiquitously active CB (cytomegalovirus enhancer/chicken β-actin promoter) regulatory cassette (AAV-CBhGAA) provoked both T cell and antibody responses against GAA and failed to achieve biochemical correction in immunocompetent GAAKO mice.[3] In contrast, liver-specific expression of hGAA with an adeno-associated vector (AAV-LSPhGAA) has established immune tolerance in GAAKO mice, as demonstrated by the absence of antibody formation in response to a subsequent immune challenge with rhGAA and adjuvant.[6,7] Pompe disease patients who lack any residual GAA protein are deemed cross-reacting immune material-negative (CRIM-negative). The relevance of antibody formation to efficacy of therapy in Pompe disease has been emphasized by the poor response of CRIM-negative patients to enzyme replacement therapy

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(ERT), which correlated with the onset of high-titer antibodies.[8]

The antibody responses in GAAKO mice and in CRIM-negative Pompe disease patients stem from the complete absence of GAA expression, because the immune system will recognize GAA as a foreign protein. For instance, in GAAKO mice, the formation of anti-GAA antibodies and hypersensitivity reactions prevented continuation of ERT beyond 3 weeks.[6,9] Long-term ERT could be tested in a Pompe disease mouse model only by the generation of liver-expressing transgenic Pompe disease mice that were immune tolerant to GAA.[9] Fortunately, hypersensitivity reactions have been absent or managed medically in patients with Pompe disease.[10]

The immune mechanisms for the different efficacy by AAV-CBhGAA or AAV-LSPhGAA vectors remain largely unknown. We have currently investigated acute immune responses in GAAKO mice induced by a liver-expressing, tolerogenic vector (AAV-LSPhGAA) or a ubiquitously-expressing, immunogenic vector (AAV-CBhGAA),[3,11] including the role of Toll-like receptors in modulating innate and adaptive immune responses.

Materials and Methods

Preparation of AAV 2/8 vectors

Briefly, 293 cells were transfected with the pAAV-LSPhGAA vector or pAAV-CBhGAA vector plasmid,[3] the AAV8 packaging plasmid (courtesy of Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA), and pAdHelper (Stratagene); thereafter, the vector was purified as described.[12] The LSP regulatory cassette (subcloned from pAV-LSP-cFIX, courtesy of Dr. Inder Verma, Salk Institute; sequence available upon request) contains a thyroid hormone-binding globulin promoter sequence downstream from two copies of an α1-microglobulin/bikunin enhancer sequence, and previously achieved long-term efficacy in hemophilia B mice within an AAV vector encoding the coagulation factor IX.[13]

In vivo analysis of AAV vector

The AAV vector stocks were administered intravenously (via the retro-orbital sinus) in 3-month-old mice. At the indicated time points postinjection, plasma or tissue samples were obtained and processed as described below. GAA activity and glycogen content were analyzed as described.[14] MyD88KO and TRIFKO mice were kindly provided by Dr. Shizuo Akira. MyD88 heterozygous (Het)/TRIFHet mice on a C57/BL6 background were bred to generate MyD88KO as described[15] with the addition of TRIF genotyping to produce TRIFKO mice (wild-type sense, 5′-CAGGACCTCAGCC TCT CATTATT-3′; mutant sense, 5′-CTGTCCACATAGAG GATT CAGATTG-3′; and common antisense, 5′-CTAAAGCG CATG CTCCAGACTGCCTTG-3′).[16] All animal procedures were done in accordance with the Duke University Institutional Animal Care and Use Committee-approved guidelines.

Quantification of vector RNA and DNA

Real-time polymerase chain reaction (PCR) was performed using SYBR green in a LightCycler 480II (Roche) following the manufacturer's instructions. For reverse transcriptase (RT)-PCR, total RNA was isolated from the spleen or liver using TRIZOL. The RNA was reverse transcribed with M-MLV reverse transcriptase (Life Technologies, Inc.) and random hexamers (Invitrogen) in accordance with the manufacturer's protocol. One microliter of cDNA was used for RT-PCR. Primers used were described previously for mouse CXCL-1, interferon (IFN)-γ, and β-actin.[11] Relative mRNA expression was normalized with β-actin and calculated using the $\Delta\Delta$Ct method. Quantification of vector DNA was performed as follows using primers for human GAA and mouse β-actin. Plasmid DNA corresponding to 0.01 copy to 10 copies of human GAA gene (in 50 ng genomic DNA) was used in a standard curve. To determine the viral copy number, the $\Delta\Delta$Ct values of samples were compared to the standard curve.

Antibody quantification

The enzyme-linked immunosorbent assay (ELISA) for anti-GAA IgG was performed as described.[17] Briefly, rhGAA (5 μg) in a carbonate buffer was coated onto each well of a 96-well plate (Costar cat. no. 3596; Corning Life Sciences) at 4°C overnight. After a wash with phosphate-buffered saline (PBS) containing 0.05% Tween 20, serial dilutions of plasma samples were added in duplicate to rhGAA-coated plates and incubated at room temperature. The wells were washed with 0.05% Tween 20 + PBS, incubated with a 1:2500 dilution of alkaline phosphatase-conjugated sheep anti-mouse IgG1 at room temperature for 1 h, and washed, and an alkaline phosphatase substrate (p-nitrophenyl phosphate) was added. The absorbance at 411 nm was measured with a Tecan SpectraFluor (MTX Lab Systems) microplate reader. All samples yielded absorbance values that were within the linear range of the assay at their respective dilutions. Absorbance values were deemed positive if both values at any given serial dilution were >0.1.

Serum and liver Cxcl-1 assay

Liver homogenates were prepared as previously described.[2] Cxcl-1 in serum and liver extract was analyzed by the mouse CXCL-1/KC kit (R&D Systems) following manufacturer's instructions. The protein concentration in liver extract was quantified via the Bradford assay. The CXCL-1 concentration in liver extract was normalized to the protein concentration.

Detection of CD4+ lymphocytes in liver

Immunohistochemical detection of CD4+ lymphocytes in the liver was performed as follows. Briefly, frozen sections of liver (10 μm) were fixed with cold acetone, air-dried, and quenched with H_2O_2. After blocking with rabbit serum for 1 h at room temperature, sections were incubated with a rabbit anti-mouse CD4 antibody (BD Pharmingen, Cat. No. 550280) at a dilution of 1:50 overnight at 4°C (BD Biosciences Pharmingen). The biotinylated secondary antibody was diluted at 1:500 for incubation, and chromagen staining was carried out with VECTASTAIN elite ABC reagents according to instructions with the kit (Vector Laboratories). The sections were counterstained with hematoxylin, rinsed with bluing agent, dehydrated, and mounted permanently for photographing.

Adoptive transfer of CD4$^+$ T cells into syngeneic wild-type mice

AAV2/8-LSPhGAA and AAV2/8-CBhGAA[3] (1×10^{11} vector particles [vp]) were injected intravenously in 2-month-old

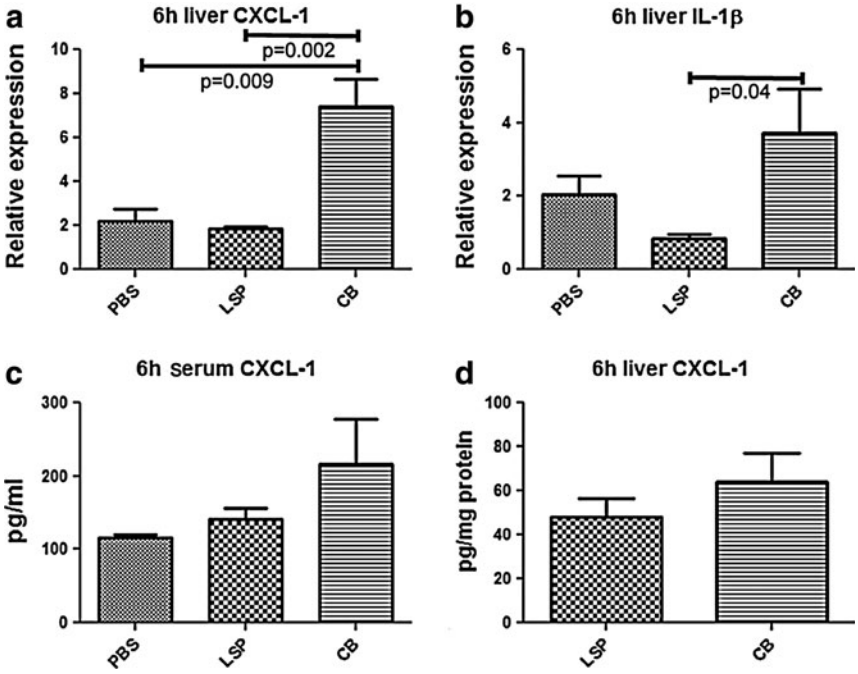

FIG. 1. An acute immune response to AAV2/8-CBhGAA. Groups of GAAKO mice (5 or 6 mice per group) were injected with AAV2/8-CBhGAA. Six hours later, serum, liver, and spleen were collected. Liver gene expression of CXCL-1 **(a)** and IL-1β **(b)** was analyzed by real-time polymerase chain reaction. CXCL-1 in serum **(c)** and liver extract **(d)** was quantified by an enzyme-linked immunosorbent assay.

MyD88KO or MyD88Het mice. After 6 weeks, $CD4^+$ T cells were isolated from spleens of mice by magnetic activated cells sorting (MACS; Miltenyi Biotec). Two-month-old syngeneic wild-type C57/BL6 mice were injected intravenously (via the retro-orbital sinus) with $2–4\times10^6$ $CD4^+$ T cells that were suspended in 100 μL PBS. The following day, the recipient mice were injected with AAV-CBhGAA (1×10^{11} vp). Six weeks later, plasma was collected for the antibody assay before they were immunized with rhGAA by an intraperitoneal injection. (2 mg/kg). Two weeks following immunization, plasma and tissue were collected.

Results

The innate response to AAV vectors encoding GAA was evaluated by monitoring cytokines during the first day following vector administration. Previously, these vectors expressed GAA in the first day following administration[3] and provoked different cytokine responses.[11] Three groups of mice were injected with AAV2/8-LSPhGAA or AAV2/8-CBhGAA (1×10^{11} vp), or untreated. After one, 6, and 24 h, serum, liver, and spleen were collected. Gene expression of a broad range of cytokines and chemokines was analyzed by quantitative RT-PCR of liver and spleen. AAV-CBhGAA induced a nearly fourfold increase in expression of CXCL-1 in liver and a 4.5-fold increase in expression of IL-1β in liver at 6 h, in comparison with AAV-LSPhGAA (Fig. 1a, b). At 1 and 24 h, AAV-CBhGAA-injected mice showed similar levels of expression in comparison with AAV-LSPhGAA-injected or untreated (Mock) groups. No change was detected in gene expression in spleen (data not shown). Mice injected with AAV-CBhGAA showed a nonsignficant twofold increase in serum CXCL-1 at 6 h following vector administration, in comparison with AAV-LSPhGAA (Fig. 1c). However, CXCL-1 expression in liver was similar following AAV-CBhGAA and AAV-LSPhGAA administration (Fig. 1d), indicating the source of CXCL-1 was from a different tissue.

It has been shown that the innate immune responses to AAV2 vectors are primarily through the TLR9-MyD88 pathway.[18] The role of MyD88/TRIF signaling pathway in shaping the antibody responses to the AAV-CBhGAA vector was unknown, and therefore we investigated how MyD88/TRIF signaling affects antibody responses to AAV2/8-CBhGAA. AAV-CBhGAA was administered to MyD88KO mice, TRIFKO mice, TRIFHet mice, and MyD88Het/TRIFHet mice. Plasma was collected at 3 and 6 weeks and anti-GAA IgG1 was analyzed by Elisa. Het/Het mice developed robust anti-GAA IgG1 responses at 6 weeks (Fig. 2a). Thus, we considered the Het/Het mice same as wild-type controls. TRIFKO mice had decreased anti-GAA IgG1 responses at 3 weeks ($p<0.05$). TRIFHet mice had an intermediate level anti-GAA IgG1 compared with TRIFKO and TRIF wild-type (WT) mice. Importantly, MyD88KO mice had diminished anti-GAA IgG1 responses at 6 weeks ($p=0.02$). Vector genomes were significantly higher in the liver of MyD88KO mice than in Het/Het mice ($p<0.001$) (Fig. 2b). Liver GAA in MyD88KO mice was much higher than in WT mice (Fig. 2c). Immunohistochemistry in the liver showed increased $CD4^+$ T cells in MyD88KO mice (Fig. 3), suggesting that increased $CD4^+$ T cells are associated with diminished anti-GAA IgG1 responses.

Next, we tested whether $CD4^+$ T cells from AAV-CBhGAA-injected MyD88KO mice are responsible for suppressing antibody responses to GAA, we performed an adoptive transfer experiment as shown (Fig. 4). MyD88KO mice or WT mice were injected with AAV-CBhGAA. Six weeks later, $CD4^+$ T cells were isolated and transferred to MyD88 Het recipients. One day following cell transfer, mice were injected with AAV2/8-CBhGAA. Plasma was collected in recipients 6 weeks later. Compared with recipients of MyD88KOHet $CD4^+$ T cells ($n=5$), recipients of MyD88KO

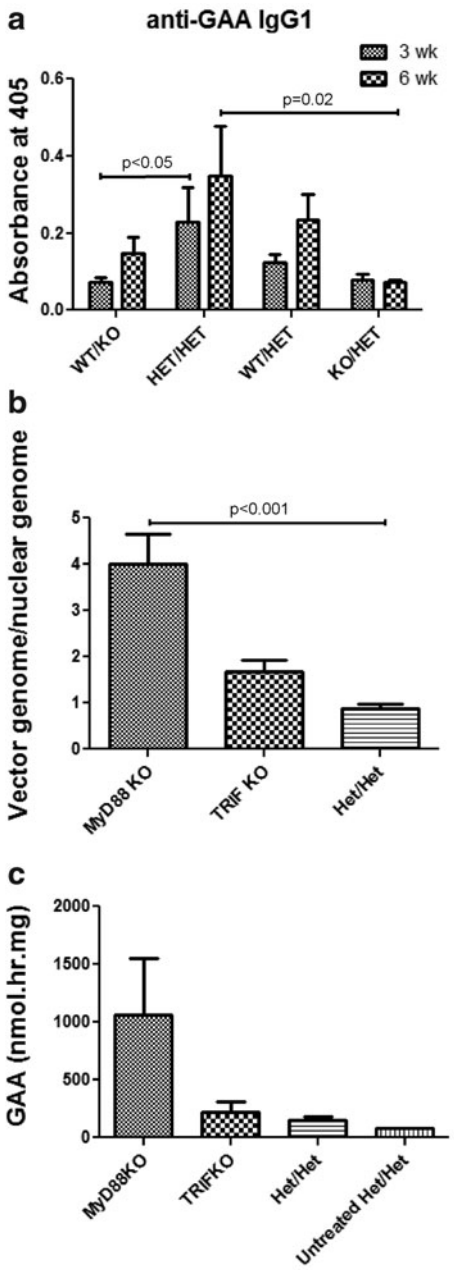

FIG. 2. MyD88 deficiency results in diminished immune responses to GAA and increased liver GAA expression. MyD88KO mice (KO/HET; $n=5$), TRIFKO mice (WT/KO; $n=5$), TRIFHet mice (WT/HET; $n=5$), and MyD88Het/TRIF-Het mice (HET/HET; $n=4$) were injected with AAV-CBhGAA. Plasma was collected at 3 and 6 weeks and assayed for anti-GAA IgG1 **(a)**. DNA was extracted from liver and was used to quantify the vector genome **(b)**. Liver extracts were prepared and assayed for GAA **(c)**.

$CD4^+$ T cells ($n=9$) had significantly decreased anti-GAA IgG1 responses ($p=0.04$) (Fig. 5a). Importantly, this pattern of the antibody response maintained following upon further immunization with rhGAA (Fig. 5b). This data were consistent with our hypothesis that $CD4^+$ T cells in MyD88KO mice injected with CB-hGAA are responsible for suppressing anti-GAA IgG1 responses. Among the transferred $CD4^+$ T cells, antigen-specific Tregs might have the role of suppressing anti-GAA IgG1 responses to AAV-CBhGAA.

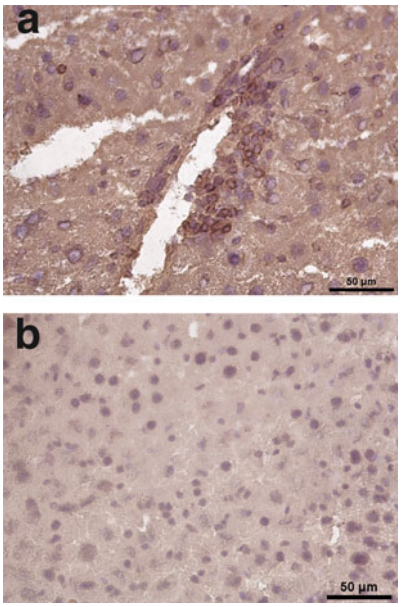

FIG. 3. Detection of CD4+ lymphocytes in the liver. Immunohistochemistry of liver sections from the above-described experiment in Figure 2. Frozen section of liver were prepared from both MyD88KO and control Het/Het mice. CD4 staining in livers from MyD88KO mice **(a)** and Het/Het mice **(b)**.

Discussion

In this study, we proved that small and transient innate immune response to AAV2/8-CBhGAA was associated with adaptive immune responses to GAA. We found that both MyD88 and TRIF signaling pathway were important for antibody responses to AAV-CBhGAA. More importantly, $CD4^+$ T cells (possibly $CD4^+$,$CD25^+$ T cells) were responsible for

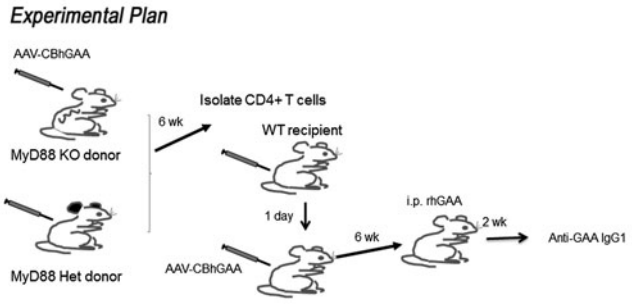

FIG. 4. Evaluation of $CD4^+$ T cells in immune tolerance in MyD88KO mice by adoptive transfer experiment. Experimental outline: MyD88KO mice were injected with AAV2/8-CBhGAA. Six week later, $CD4^+$ T cells were isolated and injected into naïve WT mice. Control mice received cells from MyD88 Het mice. The next day, all mice were given AAV-CBhGAA. Six weeks later, plasma was collected for an analysis of anti-GAA IgG1. Then, all mice were immunized by intraperitoneal injection with rhGAA. Two weeks following immunization, plasma was collected for an analysis of anti-GAA IgG1.

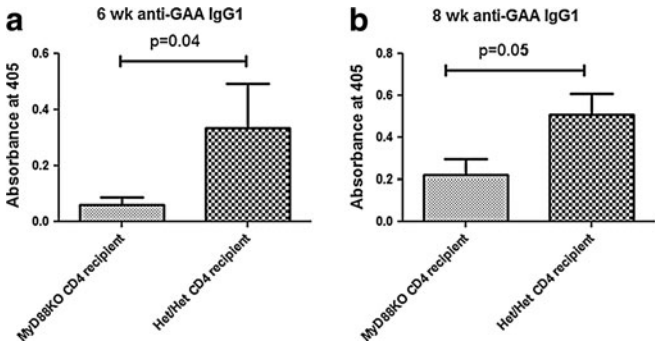

FIG. 5. The critical role of MyD88 in immune tolerance as demonstrated by adoptive transfer of $CD4^+$ T cells. MyD88KO mice were injected with AAV-CBhGAA and evaluated as shown in Figure 4. **(a)** Anti-GAA IgG1 in MyD88KO mice ($n=5$) and MyD88 het mice ($n=5$), as determined 6 weeks following vector administration. **(b)** Anti-GAA IgG1 in WT recipients from MyD88KO donors (MyD88KO CD4 recipient; $n=9$) and in WT recipients from MyD88 het donors (Het/Het CD4 recipient; $n=5$).

decreased anti-GAA IgG1 responses in MyD88KO mice receiving AAV-CBhGAA. The effect of TRIF deficiency was less than that of MyD88 deficiency, consistent with its adaptor role in the MyD88/TRIF response.[16] The current data for AAV-CBhGAA are similar to the reported patterns of acute immune responses to AAV2, although we found changes in expression of fewer genes and peaks at different times. In the past, it is generally held that innate immune responses to AAV vectors are weak and transient.[19] However, Zaiss, et al. reported that a AAV2 vector induced high expression of several inflammatory genes in liver 1h following the vector injection, similarly to an adenovirus vector, and that this increase disappeared by 24 h. A recent study showed a mild three-to fourfold increase in hepatic expression of TLR9, MyD88, TNF-α, IFN-α/β, IP-10, and CCL2 after 2 h injection of a ssAAV-F.IX via portal circulation.[20] All responses were transient and declined after 6 h. AAV8 was considered less immunogeneic than AAV2. Indeed, there was no change in many genes we tested; only the expression of CXCL-1 and IL-1β induced by CB-hGAA was detected. The TLR9-MyD88 signaling pathway was shown to be involved in activation of pDC to produce IFNα/β. The cellular source of increased CXCL-1 induced by CB-hGAA could be Kupffer cells in the liver or monocytes in circulation, while increases in IL-1β suggest activation of the inflammasome. The precise mechanism of immune activation in the liver by AAV-CBhGAA, and not AAV-LSPhGAA, remains to be further investigated.

A model for immune tolerance following gene therapy in Pompe disease recognizes the central role of regulatory T (Treg) cells in the setting of liver-specific transgene expression.[21] Several factors determine the ability to avoid immune responses against the transgene through liver-specific expression. Liver-specific expression of human FIX in mice with hemophilia B prevented antibody formation in response to an immune challenge with human FIX.[22] Furthermore, the adoptive transfer of $CD4^+$,$CD25^+$ cells (including Treg cells) to naïve recipient mice, following administration of the AAV vector-expressing human FIX to donor mice, prevented antibody formation in response to an immune challenge with FIX.[22] An AAV vector containing a liver-specific regulatory cassette to drive α-galactosidase expression-induced immune tolerance to α-galactosidase in Fabry disease mice, and the transfer of splenocytes from vector-treated mice prevented the antibody response against an α-galactosidase challenge in recipient Fabry mice.[23] Finally, anti-CD25 administration prevented the induction of immune tolerance with our LSP-containing vector in GAAKO mice, presumably be depleting Treg cells.[7] Taken together, these data strongly support the ability of an AAV vector containing a liver-specific regulatory cassette to induce immune tolerance to an introduced foreign protein.[6,7]

The importance of antibody formation in response to therapy in lysosomal storage disorders, such as Pompe disease, has become increasingly evident. The formation of high titer, sustained antibodies has correlated strongly with clinical decline and abbreviated survival, despite compliance with standard of care ERT.[24] Furthermore, the clinical relevance of antibody responses to ERT in other lysosomal storage disorders has been increasingly apparent.[25] Thus, further characterization of the antibody responses to ERT and development of therapeutic strategies to address these responses is warranted.

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Author Disclosure Statement

No competing financial interests exist.

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