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Mapping the T helper cell response to acid α -glucosidase in Pompe mice

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Abstract

Pompe disease is a neuromuscular disease caused by an inherited deficiency of the lysosomal enzyme acid α -glucosidase (GAA). The resulting accumulation of glycogen causes muscle weakness with the severe form of the disease resulting in death by cardiorespiratory failure in the first year of life. The only available treatment, enzyme replacement therapy (ERT) with recombinant GAA (rhGAA), is severely hampered by antibody responses that reduce efficacy and cause immunotoxicities. Currently, Pompe mice represent the only pre-clinical model for development of new treatments and for immunological studies. While antibody formation following ERT in this model has been described, the underlying T cell response has not been studied. In order to define the T helper response to rhGAA in Pompe mice, immunodominant CD4⁺ T cell epitopes were mapped in GAA^{-/-} 129SVE mice using ELISpot. Additionally, cytokine responses and antibody formation against rhGAA during ERT were measured. Among the three CD4⁺ T cell epitopes identified, only epitope IFLGPEPKSVVQ, predicted to be the strongest MHC II binder, consistently contributed to IL-4 production. Frequencies of IL-4 producing T cells were considerably higher than those of IL-17 or IFN- γ producing cells, suggesting a predominantly Th2 cell mediated response. This is further supported by IgG1 being the prevalent antibody subclass against rhGAA during ERT and consistent with prior reports on IgE formation and anaphylaxis in this model. These results will facilitate mechanistic studies of the immune response to rhGAA in Pompe mice during development of new therapies and tolerance protocols.

Keywords

alpha-Glucosidase; Pompe; Immune response; T cell; Antibody

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Author contributions

SN, RS, and OC performed experiments. SN, RS, OC, HD, BJB, and RWH designed experiments. OC, BJB, and RWH supervised the study. RS, SN, HD, BJB and RWH wrote the manuscript.

1. Introduction

Pompe disease is a debilitating neuromuscular disorder, caused by partial or complete deficiency of the lysosomal enzyme acid α -glucosidase (OMIM # 232300) (GAA). The disease is a continuum of clinical spectrums including progressive skeletal muscle weakness, cardiorespiratory failure. The recessive inheritance has been detected with varying frequencies in several ethnic groups. Severity of Pompe disease varies with the amount of residual endogenous enzyme production thereby influencing the age of and rate of progression of muscle weakness [1]. The early-onset phenotype, diagnosed within the first 6 months of life, is considered to be the most severe form of the disorder. All early onset patients are found to have severe cardiac hypertrophy, profound muscle weakness, hypotonia, and die due to cardiorespiratory failure within a year of age [2–5].

Diagnostic challenges are often encountered since as a rare disease there will be only 20–30 early onset children born in the US each year, yet more rapid diagnostic methods have shortened the time from symptom onset to definitive diagnosis [3]. The diagnosis is established by measurement of GAA activity in a blood spot assay, whole blood sample, or in skin fibroblasts. Although detection of GAA from skin fibroblasts was considered to be the gold standard, preference is being given to assays run on dried blood spots for their accuracy and rapid mode of diagnosis [3].

Therapeutic approaches, including gene and enzyme replacement therapies have been developed for replacing the deficient/dysfunctional enzyme. *In vivo* and *in vitro* studies using viral vectors, cell lines, and recombinant proteins have shown that the defect can be corrected by gene transfer to the affected cell (from autonomous expression) or from cross-correction [2,6]. In the latter case, extracellular GAA is taken up by cells via the mannose-6-phosphate receptor and traffics to the lysosome, resulting in clearance of glycogen. This mechanism has been the basis for enzyme replacement therapies (ERT) and some novel gene therapy approaches. Currently, the recombinant human enzyme, Myozyme® has been approved by the US and EU for use in infantile Pompe disease. The pivotal study of ERT in infants showed significant improvement in survival as a secondary outcome measure, however limitations in ventilator free survival, the primary outcome of the study, was observed over time.

Notably, very high doses of enzyme are required for a therapeutic effect and ERT for Pompe disease faces a major hurdle in the form of antibody formation against the intravenously infused recombinant GAA (rhGAA). The incidence of antibody formation is very high in early onset disease (>90%), negatively effects efficacy of treatment, and immunotoxicities such as allergic/anaphylactic reactions and tissue toxicities can occur [2,7–9]. Therefore, a better understanding of the immune response to rhGAA is imperative, and the development of immune tolerance protocols is desirable. Pre-clinical and translational studies in animal models will aid in these efforts.

Although there are several naturally occurring animal models for Pompe disease including the Lapland dog, cats, sheep and Brahman and Shorthorn cattle and a particular strain of Japanese quail, only the GAA^{-/-} mouse model serves as a more phenotypically and genotypically similar model for the human disease. To this end, knockout mice had been generated by several groups by targeting exons 6, 13, or 14. Generalized and progressive muscle weakness was observed in these models, along with elevated levels of lysosomal glycogen in the heart, liver and striated muscles. Therefore, Pompe mice are currently the animal model of choice for basic and translational studies toward improved therapies for the disease [10].

Pompe mice form high-titer antibodies to human GAA during ERT, including IgG and IgE, and develop fatal anaphylactic reaction after subsequent exposure [11]. Experiments in this model have clearly shown that the antibodies interfere with efficacy of treatment. CD4⁺ T cells play a critical role in B cell activation and antibody production in protein replacement therapies. However, the underlying T helper cell response that drives antibody formation in the Pompe mouse has not yet been studied. Using a Pompe mouse strain which is congenic with the 129SVE line, we mapped three CD4⁺ T cell epitopes in this study and found a response dominated by Th2.

2. Materials and methods

2.1. Animals

Pompe mice (8–12 weeks old) were generated by targeted disruption of exon 6 with the neomycin resistance gene ($6^{neo}/6^{neo}$) were used in this study [10]. With assistance by Taconic, these mice had been repeatedly backcrossed to 129SVE for over 10 generations to obtain a pure strain background. The mice were housed under specific pathogen free conditions in the Animal Facility at the University of Florida.

2.2. Peptide library

A peptide library spanning the mature rhGAA sequence of 952 amino acids was generated in the form of 12-mers with an overlap of 2 amino acids on both ends (Anaspec, San Jose, CA). The peptide stocks were dissolved using dimethylsulfoxide (DMSO) to a concentration of 2 mg/ml. Two-dimensional arrays of the peptides were designed to obtain 20 pools of peptides [12,13]. Each pool was composed of 8–10 peptides, and each peptide was represented in two pools. The final concentration of each peptide in the pool was 4 µg/ml. The reconstituted peptides were stored at –70 °C. Alternatively, to avoid the high concentration of DMSO during cell culture, peptides were diluted using 5-MLC media, and used to construct smaller pools consisting of only 3 peptides. Thus, 32 pools of 3 peptides each were created with a final concentration of each peptide in the pool at 10 µg/ml.

2.3. Immunizations and ERT

Pompe mice [$GAA^{-/-}$ (129SVE)] were immunized subcutaneously with 200 µl of rhGAA protein (Myozyme, 50 µg/mouse) and Complete Freund's Adjuvant (cFA; GIBCO/BRL, Rockville, MD) and sacrificed 10 days later for the peptide mapping experiments. To mimic ERT, Pompe mice were treated intravenously (tail vein) with 20 mg/kg of rhGAA protein once per week for 3 weeks and sacrificed 10 days later for T cell assays. Myozyme was kindly provided by Genzyme (Framingham, MA).

2.4. Antibody assays

GAA-specific antibody titers were determined from plasma samples of the immunized mice using subclass-specific ELISA for IgG1, IgG2a and IgG2b anti-rhGAA as published [7]. Briefly, plates were coated overnight with 1 µg/ml of rhGAA protein (in 1× PBS) and treated with mouse plasma samples (1:20 dilution) and detected using horseradish peroxidase conjugated secondary IgG antibodies (at 1:2000 dilution) (Roche Molecular Biochemicals, Indianapolis, IN). Standard curves were plotted using two fold dilutions of mouse immunoglobulin standards (Sigma-Aldrich, St Louis, MO) starting at 2000 ng/ml for IgG1 and IgG2a and at 500 ng/ml for the IgG2b ELISA's. The plates were developed using OPD (o-phenylen diamine substrate, Louis, MO) substrates and the antibody titers were determined by reading the optical density measurements at 450 nm. Anti-rhGAA IgE titers were also measured by ELISA using plasma samples depleted for IgG with sepharose beads as published [14]. Plasma samples containing high-titer IgG2a anti-rhGAA were assayed for

ability of antibody–antigen complex to fix complement using an ELISA sandwich assay as described by others [15]. Briefly, wells that were coated overnight with rhGAA were incubated with plasma samples for 2 h, washed and further incubated with fresh mouse complement (1:20 dilution) (Innovative Research, Peary Court Novi, MI) for an hour at 37 °C. Anti-mouse C3 conjugated with horse radish peroxidase (1:2000) (ICL, Inc, Portland, OR) was added for detection as published [14,15].

2.5. ELISpot assays

ELISpots were performed for rhGAA-specific IL-4, IL-17, and IFN- γ responses. Sterile 96 well multiscreen filter ELISpot plates (Millipore, Billerica, MA) were pre-wetted using 15 μ l of 35% ethyl alcohol to overcome the hydrophobicity of the membrane and coated overnight at 4 °C with anti IFN- γ /IL-4/IL-17 capture antibodies (BD Biosciences, San Jose, CA). Splenocytes were isolated from 129SVE mice 10–12 days after their immunizations and cultured using 5-MLC media (DMEM, sodium-pyruvate, HEPES, non-essential amino acids, 2-beta-mercaptoethanol, 1% penicillin/streptomycin and 5% mouse serum/FBS). The splenocytes were also positively selected for CD4⁺ and CD8⁺ T cell populations by magnetic cell separation (Miltenyi Biotec, CA, USA) and used for mapping the CD4⁺ T cell dominant epitopes. Plates were blocked using PBS with 5% sucrose and 1% BSA for 2 h at room temperature after the overnight Ab incubation and then used for cell culture, with 200 μ l of 10⁶ splenocytes or peripheral blood mononuclear cells. The cells were stimulated using 10 μ g/ml of the peptide pools or 1 μ g/ml individual peptides or 10 μ g/ml of the whole rhGAA protein for 14–17 h at 37 °C in 5% CO₂ for the mapping experiments (using IFN- γ ELISpots). Staphylococcal Enterotoxin B (1 μ g/100 μ l; Sigma Aldrich, St. Louis, MO), PMA-Phorbol 12-myristate 13-acetate (0.05 g/ml)/Ionomycin (1 μ g/ml; Sigma Aldrich), and recombinant mouse IFN- γ (R&D systems Minneapolis, MN) at 1 μ g/ml were also used as positive controls. DMSO or an irrelevant peptide was added to media at identical concentration for mock-stimulation (negative controls). For responses to ERT, splenocytes were stimulated with 10 μ g/ml of a specific peptide or of GAA protein. The cells were stimulated with peptides and controls at 37 °C in 5% CO₂ for 48 h for the IL-4 ELISpots and for 24 h for the IL-17 ELISpot's. The plates were then washed and plated with anti IFN- γ /IL-4/IL-17 secondary antibodies (BD Biosciences, San Jose, CA). The color development of the spots was carried out using HRP-streptavidin (BD Biosciences, San Jose, CA) and BCIP/NBT chromogen (BD Biosciences). IFN- γ /IL-4/IL-17 secreting cells were analyzed and counted with the CTL-ImmunoSpotH S5 UV analyzer (Cellular Technology, Shaker Heights, OH) [16].

2.6. In silico analysis

The immune epitope database analysis resource (IEDB, sponsored by National Institute of Allergy and Infectious Diseases) was utilized for the prediction of I-A^b MHC class II epitopes in the GAA protein sequence. The NET MHC II pan epitope prediction tool was preferred due to its performance score and its ability to predicting murine specific MHC class II epitopes. The algorithms were based on neural networks and binding affinities of the peptides with MHC class II molecules as described previously [17].

2.7. Statistics

The mean $\pm$ SD results of all the experiments were plotted using GraphPad Prism 5.0 software. Un-paired Student t tests was used for determining statistical significant values, with p values<0.05 considered to be significant.

3. Results

3.1. Identification of a dominant CD4⁺ T cell epitope

Efficient immunoglobulin class switching, strong antibody responses and development of memory B cells are typically dependent on T help by MHC II-restricted CD4⁺ T cells. This is well documented for treatment of other protein deficiencies such as hemophilia [18]. Consistent with this notion, repeated administration of rhGAA resulted in antibody formation in wild-type but not in CD4^{-/-} C57BL/6 mice (data not shown). In order to generate appropriate tools for T cell studies, we sought to identify CD4⁺ T cell epitopes. The strain of mouse used here (GAA^{-/-} 129SVE, MHC haplotype H-2^{bc}) expresses the MHC II molecule I-A^b, which by *in silico* analyses is predicted to tightly bind GAA-derived peptides containing the core sequence IFLGPEPKSVQQ (Fig. 1B). We decided to experimentally identify the immunodominant T cell epitope against the rhGAA protein in Pompe mice using peptide libraries [13,19].

A library spanning the entire mature GAA sequence of 952 amino acids was generated in the form of 12-mers with an overlap of 2 amino acids on both ends. The library was initially split into 20 pools with 10 peptides per pool and arranged in the form of a matrix, with each peptide being represented by two pools as shown in (Fig. 2A). Pompe mice were immunized with rhGAA protein in cFA. After 10 days, their splenocytes were extracted and stimulated *in vitro* with peptide pools for analysis by IFN- γ ELISpot. Initial experiments done with the 20 peptide-pools narrowed down the response to 8 potential dominant epitopes (Fig. 2B). The experiment was repeated, and splenocytes were stimulated with the single candidate peptides. Here, only peptide # 83 yielded a response (Fig. 2C). These results are in agreement with *in silico* analyses, since peptide 83 contains the predicted high-affinity core sequence (Figs. 1A and B).

3.2. Identification of two additional CD4⁺ T cell epitopes

As the initial matrix arrangement of peptide pools were large and possessed high concentrations of DMSO, the peptides were rearranged into 32 smaller pools, consisting of 3 peptides per pool, to identify epitopes which could have been missed due to competitive effects in the previous assays. Among the 32 pools of peptides, 4 emerged positive for IFN- γ secretion, including the pool that contained peptide 83 (Figs. 3A and B). In the next experiment, stimulation with single peptides derived from these pools resulted in positive responses for peptides 19, 29, and 83 (Figs. 3C and D). Identical results were obtained when peripheral blood cells instead of splenocytes were used for the assay (data not shown). ELISpot results remained positive for peptides 19, 29, and 83, when the splenocytes were depleted for CD8⁺ cells, but were negative upon depletion of CD4⁺ cells, confirming that the identified epitopes were recognized by CD4⁺ T cells (Figs. 4A–D). Interestingly, all 3 identified epitopes were clustered around in the same region of the GAA protein (amino acids 200–350, Fig. 1A).

3.3. Relative contribution of the epitopes to Th2 responses in ERT

In order to study the contribution of the identified epitopes in the immune response to ERT, Pompe mice received 3 weekly therapeutic doses of rhGAA (20 mg/kg) by intravenous (tail vein) injection, which consistently resulted in high-titer IgG1 anti-rhGAA formation (Figs. 5A and B). Less consistently and at lower titers, IgG2a was also observed as well as low-titer IgG2b (Figs. 5A and B). No evidence for the ability of antibody–rhGAA complexes to activate complement was obtained (data not shown).

Given that IgG1 formation is driven by Th2, and that others reported IgE formation (another Th2-dependent antibody), we performed ELISpots for IL-4 production to assess Th2

immunity. Stimulation with peptide 83 consistently resulted an IL-4 response, while responses to peptides 19 and 29 were also observed, albeit less consistently and typically at lower frequency (Figs. 6A–C). Three of 4 mice also showed an IL-4 response upon stimulation with GAA protein, which was not as high as when stimulated with peptide 83 (This was somewhat expected, since the protein has to be processed prior to peptide presentation by MHC). In other assays, we also found a low-frequency IL-17 response, suggesting some level of Th17 activation (Figs. 6D–E). These responses were primarily to peptide 19. In contrast to immunization with cFA, ERT did not result in a detectable IFN- γ response (Fig. 6F). In all assays performed for Fig. 6, ELISpot data from GAA-stimulated cells tracked very well with peptide responses for each individual mouse and cytokine.

4. Discussion

Immunodominant epitopes are essential for mounting effective immune responses *in vivo*. In the past decade, more attention has been focused on mapping and characterization of T cell epitopes from protein antigens. CD4⁺ T cells have been garnering additional importance due to their significant roles in the development and functioning of both the cell mediated and humoral responses of the adaptive immune system [19]. T cell assays following immunization or ERT and *in silico* analyses were all in agreement and identified peptide IFLGPEPKSVVQ to contain the dominant epitope that derived CD4⁺ T cell responses to human rhGAA in GAA^{-/-} 129SVE Pompe mice. Two additional epitopes that contribute to the response were also identified. This knowledge should facilitate future studies on T cell responses to rhGAA in gene and protein therapies in the murine model and provide important tools for T cell assays. Furthermore, peptides containing CD4⁺ T cell epitopes can be useful in the development of immune tolerance protocols, as we and others have shown in form of nasal tolerance and co-administration of peptides with immune modulatory drugs [11,18,20–24]. For example, in the right context, peptides may stimulate immune regulatory CD4⁺ T cells that actively suppress responses. Translation of this approach will depend on the ability to effectively map T cell epitopes in patients.

Continued ERT in the Pompe mice beyond three rhGAA administrations performed here leads to fatal anaphylactic reactions. Others found that Pompe mice form IgE formation in addition to IgG, suggesting involvement of the classical anaphylaxis pathway in this severe systemic allergic response [7,11]. Anaphylactoid reactions mediated by an alternative IgG-PAF (platelet activating factor) pathway cannot be ruled out as another contribution to the pathogenic antibody response, which requires further study [25]. While we did not detect circulating IgE anti-rhGAA here (data not shown), we found that prolonged administration of a lower dose of rhGAA (5 mg/kg, which did not result in anaphylaxis as rapidly) resulted in high-titer IgE formation (unpublished observations). It should also be pointed out that IgE is primarily associated with cell surfaces, e.g. of mast cells to promote histamine release, and thus is difficult to detect in circulation. Consistent with IgG1 and IgE formation, T help against rhGAA was primarily of the Th2 subset during the first month of ERT. In addition to the IL-4 response revealed by ELISpot, quantitative RT-PCR analyses identified induction of IL-13, another Th2 cytokine that promotes class-switch to IgG1 and to IgE (data not shown). ELISpot for IL-17 results and IgG2a formation (Th1-dependent antibody) suggest that additional subsets of T helper cells such as pro-inflammatory Th17 or Th1 cells are also activated by ERT, but to substantially lesser extent than Th2. In contrast, introduction of rhGAA antigen in the context of strong inflammation, as provided by cFA, can result in strong Th1 responses specific to the same three epitopes.

In conclusion, multiple epitopes contribute to the CD4⁺ T cell response to rhGAA during ERT in Pompe mice. This results in Th2 immunity that drives the formation of pathogenic antibodies, which are known to reduce efficacy, cause toxicities and potentially life-

threatening anaphylactic reactions. Knowledge of dominant CD4⁺ T cell epitopes should be useful for measurement and mechanistic studies on the immune response to rhGAA and to develop immune tolerance protocols.

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A

MGVRHPPCSHRLAVCALVSLATAALLGHILLHDFLLVPRELSGSSPVLEETHPAHQQGASRPGPRDAQAHGPRPRAVPTQCDVPPNSRFDCAFDKAIT
 QEQCEARGCCYIPAKQGLQGAQMGPWCFFPPSPSYSKLENLSSEMGTATLTRTTPTFFPKDILTRLDMMETENRLHFTIKDPANRRYEVPLETP
 HVHSRAPSPLYSVEFSEEPFGVIVRRQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPTGANLYGSHPFYL
 ALEDGGSAGHGVFLLNSNAMDVVLQSPALSWRS TGGILDVYIFLGPEPKSVVQYLDVVGYPFMPYWGFGHLCRWGYSSTAIRQVVENMTRAHFP
 LDVQWNLDDYMSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRPYDEGLRRGVFITNETGQPLIGKWWPGSTAFPDFTNPTA
 LAWWEDMVAEFHDQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVGGTLQAATICASSHQFLSTHYNLHNLGLTEAIAASHRALVKARGT
 RPFVISRSTFAGHGRYAGHWTGDVWSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPMRNHNSLLSLPQEPYSFSEPA
 QQAMRKALTLRYALLPHLYTLFHQAHAAGETVARPLFLEFPKDSSTWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPEALGSLPPPPA
 APREPAIHSEGGQWVTLAPLDTINVHLRAGYIIPLQGGPLTTTESRQQPMALAVALTKGGEARGELFWDDGESLEVLERGAYTQVIFLARNTTIVNELVRV
 TSEGAGLQLQKVTLGVATAPQQVLSNGVPVSNFTYSPDTKVLDCVSLLMGEQFLVSWC

B

Allele	position	peptide	core	1-log50k (aff)	affinity (nM)	Bind Level
H-2-IAb	338	VYIFLGPEPKSVVQQ	FLGPEPKSV	0.6606	39.4	SB
H-2-IAb	337	DVYIFLGPEPKSVVQ	FLGPEPKSV	0.6573	40.8	SB
H-2-IAb	336	LDVYIFLGPEPKSVV	FLGPEPKSV	0.6492	44.5	SB
H-2-IAb	335	ILDVYIFLGPEPKSV	FLGPEPKSV	0.6288	55.5	WB
H-2-IAb	475	LIGKWWPGSTAFPDF	KVWPGSTAF	0.6185	62.1	WB
H-2-IAb	476	IGKWWPGSTAFPDF	KVWPGSTAF	0.6138	65.3	WB
H-2-IAb	339	YIFLGPEPKSVVQQY	FLGPEPKSV	0.6115	66.9	WB
H-2-IAb	781	ALGSLPPPPAAPREP	SLPPPPAAP	0.6094	68.5	WB
H-2-IAb	474	PLIGKWWPGSTAFPD	KVWPGSTAF	0.6084	69.2	WB
H-2-IAb	142	SSEMGTATLTRTTP	YTATLTRTT	0.6072	70.1	WB
H-2-IAb	780	EALGSLPPPPAAPRE	SLPPPPAAP	0.6065	70.7	WB
H-2-IAb	143	SEMGYTATLTRTPT	YTATLTRTT	0.6056	71.4	WB
H-2-IAb	779	VEALGSLPPPPAAPR	SLPPPPAAP	0.6033	73.1	WB
H-2-IAb	141	SSSEMGTATLTRTT	YTATLTRTT	0.6025	73.8	WB
H-2-IAb	477	GKWWPGSTAFPDFTN	KVWPGSTAF	0.6004	75.5	WB
H-2-IAb	778	FVEALGSLPPPPAAP	SLPPPPAAP	0.5931	81.7	WB
H-2-IAb	144	EMGYTATLTRTPTF	YTATLTRTT	0.5927	82.0	WB

Fig. 1.

GAA primary amino acid sequence and MHC II epitope prediction. A. Primary amino acid sequence of the lysosomal protein, acid α -glucosidase. Yellow highlights represent sequences, which were subsequently identified to contain CD4⁺ T cell epitopes: peptide 19 (HSRAPSPLYSVE), peptide 29 (VLQSPALSWRS), and peptide 83 (IFLGPEPKSVVQ). B. Prediction of the immunodominant I-A^b-restricted epitope by NET MHC II, a T cell epitope prediction server of the immune epitope database analysis source. Protein was loaded in the Fasta format and sequence IFLGPEPKSVVQ highlighted in yellow was identified as the strongest binder based on its threshold values (strong binder threshold 50.00, weak binder threshold 500.00).

Fig. 2. Initial identification of a dominant T cell epitope using a peptide library. Pompe mice (GAA^{-/-} 129SVE) were immunized with rhGAA/CFA followed by *in vitro* stimulation of splenocytes with peptides and IFN- γ ELISpot. A. Matrix of 20 peptide pools, each containing 10 peptides with each peptide being represented in 2 pools. Candidate peptides that emerged from the assay are indicated. B. Average frequencies of IFN- γ spot forming units ($\pm$ SD) obtained for each pool. C. Average frequencies of IFN- γ spot forming units ($\pm$ SD) obtained for stimulation with individual candidate peptides. All stimulations were performed in triplicate (using splenocytes pooled from 3 animals). Control stimulations included mock (adding an irrelevant peptide or an identical amount of DMSO solvent to the media), SEB superantigen, and rhGAA protein.

Fig. 3. Identification of additional dominant T cell epitopes for rhGAA in Pompe mice. A. Representative examples of well images from IFN- γ ELISpot assays after stimulation of splenocytes with small peptide pools, individual peptides, and controls. GAA^{-/-} 129Sv mice had been immunized with rhGAA/CFA. B. Average frequencies of IFN- γ spot forming units ($\pm$ SD, triplicate) after stimulation with small peptide pools or C. Individual peptides derived from pools that had tested positive.

Fig. 4.

Confirmation that identified peptides contain CD4⁺ T cell epitopes. A. Representative examples of well images from IFN- γ ELISpot assays after stimulation of CD4⁺ cell-depleted splenocytes with peptides 19, 29, 83, rhGAA protein, SEB or solvent control. GAA^{-/-} 129SVE had been immunized with rhGAA/CFA. B. Average frequencies of IFN- γ spot forming units ($\pm$ SD, triplicate) after CD4⁺ depletion and stimulation with indicated peptides or controls. C. Representative examples of well images from IFN- γ ELISpot assays after stimulation of CD8⁺ cell-depleted splenocytes. D. Average frequencies of IFN- γ spot forming units ($\pm$ SD, triplicate) after CD8⁺ depletion and stimulation with indicated peptides or controls.

Fig. 5. Antibody formation against rhGAA during ERT in Pompe mice. GAA 129SVE mice were treated with 3 weekly intravenous doses of rhGAA (20 mg/kg). One week later, plasma samples were collected, and rhGAA-specific IgG titers were measured. A. GAA-specific IgG1, IgG2a, and IgG2b titers for 8 individual animals. B. Average titers ($\pm$ SD) for the 3 subclasses. Statistically significant differences are indicated as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$.

Fig. 6.

Cytokine responses to the immunodominant epitopes upon ERT in Pompe mice. $GAA^{-/-}$ 129SVE mice received 3 weekly intravenous doses of rhGAA (20 mg/kg) followed by splenocyte assays. **A.** Representative examples of well images IL-4 ELISpot assay after stimulation with peptide 19, 29, or 83, or GAA protein, or SEB, or mock controls. **B.** Average frequencies of IL-4 spot forming units for 4 mice assayed individually (duplicate stimulations). **C.** Average values ($\pm$ SD) for the group of 4 animals. Peptide 83 showed statistically significant responses compared to mock stimulation. **D.** Average frequencies of IL-17 spot forming units for 3 mice assayed individually (duplicate stimulations). **E.** Average values ($\pm$ SD) for the group of 3 animals. Peptide 19 showed statistically significant

responses compared to mock stimulation. F. Average values ($\pm$ SD) for 4 animals assayed by IFN- γ ELISpot.